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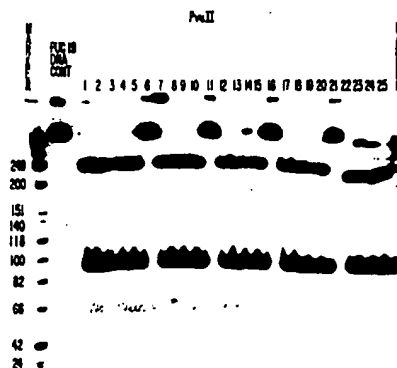
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**D-80504 München (DE)**(54) **Method for suppressing inhibition of enzyme-mediated reactions by ionic detergents.**

(57) Methods and kits for the use of a non-ionic detergent to suppress enzyme inhibition in a reaction solution due to the presence of inhibiting ionic detergent. Prior to reaction, the reaction mixture is given an effective amount of a non-ionic detergent, and agitated. The enzyme is then added, and the enzymatic reaction is the allowed to proceed. Also disclosed are preferred embodiments of the present invention, and kits for nucleic acid amplification of a biological sample in the presence of an ionic detergent.

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## TECHNICAL FIELD OF THE INVENTION

The present invention relates to methods for conducting enzymatic reactions in the presence of ionic detergents, for example lithium lauryl sulphate (LLS) and sodium dodecyl sulfate (SDS), which are often present in diagnostic and clinical samples as solubilizing and protein denaturing agents. This invention thereby eliminates the necessity for lengthy and labor-intensive procedures to separate the detergent from an analyte or an enzyme substrate before initiating the desired reaction. The present invention further relates to a method for conducting enzyme-mediated nucleic acid amplification reactions such as the polymerase chain reaction (PCR) or restriction enzyme digests in the presence of anionic detergents such as LLS without the need for a detergent removal step.

## BACKGROUND OF THE INVENTION

This invention concerns enzyme-mediated chemical reactions conducted in vitro, and techniques for preventing their inhibition in the presence of detergents. Detergents are common tools in medical and biological research laboratories, primarily due to their ability to solubilize various proteins, cell wall and membrane components, and other cellular organelles, substructures, and components which are either insoluble or incompletely soluble in aqueous solution alone. Thus, detergents are often components of extraction or lysis buffers, both as lytic agents and as effective inhibitors of undesired enzyme activities such as those contributed by the proteases and nucleases normally present in a crude cell lysate. Additionally, such detergents are often used for the same purposes in the purification of nucleic acids.

Most enzymes used as tools in applied molecular and biological chemistry are quite sensitive to detergents, especially strong ionic detergents such as sodium dodecyl sulphate (SDS) or lithium lauryl sulphate (LLS). Such ionic detergents can bind strongly to proteins, often resulting in irreversible denaturation of the protein. (See American Society for Microbiology, Manual of Methods for General Bacteriology 57-58 (1981)). However, for precisely this reason ionic detergents such as LLS are often an extremely valuable and inexpensive short- to medium-term preservative of nucleic acids in solution. Thus, such agents are useful to assist in accomplishing the first step of a nucleic acid hybridization assay using microorganisms; extraction of the nucleic acids from microbial cells or particles. Ionic detergents help to solubilize the cell wall and cell membrane, and to simultaneously prevent degradation of the nucleic acids by nucleases. (See id.) Moreover, strong ionic detergents such as SDS or LLS are often added to the lysis, permeabilization, or transport media in which clinical specimens are conveyed to the laboratory for analysis.

Often nucleic acids obtained from a biological sample are subsequently subjected to enzymatic manipulation, such as digestion with a restriction endonuclease or an exonuclease specific to DNA or RNA. Additionally, nucleic acids obtained from such samples are often not present in amounts large enough for them to be directly detected and/or quantified by nucleic acid hybridization techniques. Thus, the nucleic acid sequences of interest in such samples must normally be enzymatically amplified to be detected.

In biological or clinical samples to be subjected to one or more rounds of nucleic acid amplification or another enzyme-mediated reaction, the detergent must be separated from the nucleic acids in solution before an enzyme can be added to the reaction mixture. Dialysis or ultrafiltration, which usually works well to remove small molecules from a solution will not effectively remove most detergents, probably due both to the size of the micelles formed by the aggregation of the detergent molecules, as well as ionic or hydrophobic binding of the detergent molecules to larger solutes. Moreover, neither dialysis nor ultrafiltration is conveniently adaptable for use in a commercial diagnostic kit.

It would be convenient and cost-effective to perform an enzyme-mediated reaction such as nucleic acid amplification or a restriction digest in the same tube or collection vessel as is used to transport the biological sample to the laboratory for analysis, i.e. in the presence of SDS or LLS. Alternatively, it would be desirable to conduct such a reaction using such a sample as the immediate starting material, rather than having to subject the sample to an additional detergent-removing step. Although SDS can be precipitated with solvents such as acetone, acetone can denature or precipitate some enzymes. Moreover, the desired reaction may be inhibited by traces of acetone or other precipitating agents.

Currently, nucleic acids in a crude sample are generally purified prior to conducting an amplification by means of a phenol/chloroform extraction and subsequent ethanol precipitation. The method of the present invention takes advantage of both the similarities and the differences between ionic and non-ionic detergents to eliminate the necessity for such a step, thereby allowing enzyme-mediated reactions to be performed using nucleic acids in a biological sample, even when the sample contains an amount of ionic detergent which would normally inhibit the reaction.

The present invention is preferably a method for performing a nucleic acid amplification reaction, such as the polymerase chain reaction (PCR) or a transcription-based amplification system, in the presence of anionic detergents such as sodium dodecyl sulphate (SDS) or lithium lauryl sulphate (LLS). However, this present invention should be capable of preventing the inhibition of other enzymatic reactions, such as restriction digests, endo- and exonuclease digests, and kinase and transferase reactions by ionic detergents as well. Nor does the Applicant contemplate that the application of the present invention is limited to enzymatic reactions involving nucleic acids. Thus, while the embodiments of the present invention contained herein illustrate the use of the present invention in amplification reactions, such embodiments are meant to be exemplary only, the scope of the present invention being defined solely by the claims with which this specification concludes.

While not wishing to be bound by theory, Applicants believe that the formation of colloidal aggregates comprising heterogeneous micelles of non-ionic and ionic detergent molecules effectively remove the ionic detergent molecules from solution, thus making them unavailable to bind with or denature the subsequently added enzyme.

The ability of detergents to enhance or restore the activity of some enzymes has been reported. Saito, M., et al., Action of Arthrobacter ureafaciens Sialidase on Sialoglycolipid Substrates, 254 *J. Biol. Chem.* 7845-54 (1979). The use of heterogeneous micelles of ionic and non-ionic detergents as a method for the reactivation of detergent-inhibited proteins has also been reported. See Ey, P.L. & Ferber, E., Calf Thymus Alkaline Phosphatase II. Interaction with Detergents, 480 *Biochim. Biophys. Acta* 163-77 (1977); Berge, R.K., et al., Variations in the Activity of Microsomal Palmitoyl-CoA Hydrolase in Mixed Micelle Solutions of Palmitoyl-CoA and Non-Ionic Detergents of the Triton X Series, 666 *Biochim. Biophys. Acta* 25-35 (1981); Tandon S., & Horowitz, P.M., Detergent-assisted Refolding of Guanidinium Chloride-denatured Rhodanese, 262 *J. Biol. Chem.* 4486-91 (1987). Additionally, the formation of heterogeneous micelles of ionic and non-ionic detergents has been reported as a method for removing inhibiting concentrations of non-ionic detergents from a solubilized enzyme preparation. Stralfors, P. et al., Removal of Nonionic Detergent from Proteins Fractionated by Electrofocusing, 533 *Biochim. Biophys. Acta* 90-97 (1978).

All of the methods mentioned in the publications listed above involve the activation or reactivation of enzymes in a detergent solution; none of these methods teach or suggest the formation of heterogeneous micelles in a solution containing an enzyme substrate before the addition of active enzyme. Additionally, in all these cases detergents were used to solubilize, purify, or activate the enzyme; in no case was the detergent initially added to solubilize and stabilize the enzyme substrate rather than the enzyme itself.

#### DEFINITIONS

In this application the following terms have the following meanings, unless expressly stated to the contrary herein.

By "substantial inhibition" and "substantially inhibit" is meant a decrease in enzyme activity below that acceptable for reliable, sensitive and reproducible assays of enzyme activity.

By "target nucleic acid sequence", "target nucleotide sequence" or "target sequence" is meant a specific nucleic acid sequence, or the nucleic acid sequence complementary thereto.

By "heterogeneous micelles" is meant hydrophobic aggregates comprising monomers of ionic and non-ionic detergent molecules in a liquid medium.

By "mixed micelles" is meant "heterogeneous micelles", as defined in this disclosure.

By "complementary" is meant having a nucleic acid sequence whereby stable hydrogen bonds are formed between the nucleotide bases of a region of one nucleic acid strand and those of a region of another nucleic acid strand under conditions suitable for discriminatory nucleic acid hybridization. That is, hydrogen bonds are most commonly formed between an adenosine(A) residue on one strand and a thymine(T) or uracil(U) residue on another strand, and between a guanine(G) residue on one strand and a cytosine(C) residue on another strand. Such regions of complementarity generally involve between about 12 and 100 or more contiguous nucleotides of each nucleic acid strand.

By "sufficiently complementary" is meant capable of forming a double-stranded hydrogen-bonded region with a target nucleic acid under hybridization conditions suitable to prevent a non-complementary nucleic acid from hybridizing thereto. While two nucleic acid strands are sufficiently complementary if they have 100% complementarity over specific contiguous and corresponding regions, it is known to those skilled in the art that two single stranded nucleic acids having regions of less than 100% complementarity can form a double-stranded region under selective hybridization conditions. Such regions, not 100% complementary but able to form stable double stranded regions under these hybridization conditions, are hereby considered sufficiently complementary.

By "analogous" is meant a single-stranded nucleic acid region having a nucleotide sequence identical or similar to that of a second single-stranded nucleic acid to which it is being compared. This includes, for example, nucleic acids wherein the first nucleic acid contains a uracil residue in said region in place of a thymine present in the second nucleic acid and nucleic acid regions encoding or comprising functionally similar or identical biological agents or parts thereof.

By "sufficiently analogous" is meant having a nucleic acid sequence, as compared to a first single stranded nucleic acid, which allows a second single-stranded nucleic acid having that sequence to form a stable, hydrogen-bonded double-stranded region with a third nucleic acid under nucleic acid hybridization conditions, and wherein the third nucleic acid is sufficiently complementary to the first nucleic acid.

By "RNase-inhibiting agent" is meant any agent capable of preventing the degradation of RNA by enzymes having RNase activity. The term includes but is not limited to: enzymes such as proteases, cross-linking reagents, antibodies, and compounds which block the active site of the RNase molecule.

By "nucleic acid" is meant polydeoxyribonucleotides or polyribonucleotides of at least two, and preferably 10 or more nucleotides in length. The term "nucleic acid" includes polynucleotides, oligonucleotides, and DNA or RNA molecules. The term "nucleic acid" can refer to either single-stranded or double-stranded polynucleotides, or both.

By "target nucleic acid" is meant a nucleic acid comprising a target nucleic acid sequence.

By "biological sample" or "test sample" is meant any specimen or sample containing substances derived at any time from living organisms containing nucleic acids. Such samples include, but are not limited to, food or agricultural samples; environmental samples; samples containing body fluids, secretions or excretions such as urine, blood, milk, cerebrospinal fluid, sputum, saliva, stool, lung aspirates, tears, lymphatic fluid, or semen; throat or genital swabs; and bacterial, viral, plant or animal cell cultures, suspensions or lysates.

By "amplification" or "target amplification" is meant increasing the number of target nucleic acid molecules having a target nucleic acid sequence.

Methods for detecting nucleic acids are well known in the art, and generally consist of contacting at least one labeled single-stranded nucleic acid probe with a single-stranded target nucleic acid under hybridization conditions, where the probe has a nucleic acid sequence sufficiently complementary to that of the target nucleic acid and the target nucleic acid has a specific sequence the presence of which is desired to be known; such methods are described in Maniatis, T., et al., Molecular Cloning: A Laboratory Manual - (Cold Springs Harbor Laboratory 1982). Detection of double-stranded hybrid molecules depends on the nature of the label used; generally the probe incorporates a radioactive isotope such as  $^{32}\text{P}$ ,  $^3\text{H}$ , or  $^{14}\text{C}$ , or is conjugated with a fluorescent moiety, a hapten, or another non-radioactive reporter group. See e.g., Maniatis, *supra*, and Arnold et al., PCT US88/02746, both of which are hereby incorporated by reference as part of this disclosure.

The sensitivity and reliability of diagnostic nucleic acid hybridization can be improved by using any one of a number of enzyme-mediated amplification systems to increase the copy number of the target nucleic acid sequence. Generally, such methods use the target nucleic acid as a template for at least one nucleic acid polymerase which may be used in concert with two or more nucleic acid primers in reiterative cycles to provide an exponential increase in the number of target nucleic acid sequences. Examples of amplification systems that are well known to those skilled in the art include the polymerase chain reaction (PCR) (Mullis et al., 155 Methods in Enzymology 335-50 (1987) and use of the double-stranded PCR products as templates for making multiple single-stranded RNA transcripts (Murakawa et al., 7 DNA 287-95 (1988)). Other amplification systems involve alternate rounds of DNA synthesis on an RNA template and transcription to amplify the target sequence. See e.g., Burg et al., WO 89/1050; Gingeras et al., WO 88/10315; Kacian and Fultz, EPO Application No. 89313, both of which are incorporated by reference as part of this disclosure. The preceding list is not intended to be an exhaustive listing of the various amplification methods, and other nucleic acid polymerases suitable for use in a nucleic acid amplification system are known to those skilled in the art. Following amplification, the resulting amplified nucleic acids can then be identified using a detection system as mentioned above.

Thus in a first aspect, the present method features a method for preventing an ionic detergent from denaturing or otherwise substantially inhibiting the activity of enzymes used in nucleic acid amplification reactions comprising: 1) target nucleic acids, 2) one or more nucleic acid primers, each one sufficiently complementary to a common target nucleic acid sequence to form double-stranded hydrogen-bonded regions with the target under suitably selective hybridization conditions, 3) necessary nucleoside triphosphates, salts, and other components necessary to achieve amplification, and 4) a quantity of an ionic detergent. The method of the present invention does not require a detergent removal step such as dialysis or chromatography. The method involves the addition of a non-ionic detergent to the sample and agitation

of the sample in order to form mixed micelles of ionic and non-ionic detergent molecules. Following the formation of heterogeneous micelles, the nucleic acid polymerase and any other enzymes needed to conduct amplification can be added to the sample and the nucleic acid amplification reaction performed as usual.

5 In a second aspect of the present invention, a biological sample containing microorganisms or nucleic acids is contacted with a lysis or extraction buffer, and the released nucleic acids are stored in a buffer containing an ionic detergent such as LLS or SDS for subsequent amplification and use in a nucleic acid detection assay. Prior to the amplification step, a quantity of a non-ionic detergent is added to the extracted nucleic acid mixture either simultaneously with or preceding the addition of an effective quantity of ribo- or  
10 deoxyribonucleotide triphosphates, generally two or more nucleic acid primers, and any co-factors or metal ions necessary for the enzymatic reaction. The solution is then mixed vigorously, after which a nucleic acid polymerase is added to the mixture. The reaction mixture is incubated as appropriate for the amplification method used, for example a thermocycling or an isothermal amplification protocol. See e.g., American Society for Microbiology, *Diagnostic Molecular Microbiology: Principles and Applications* 56-70(1993), which  
15 is hereby incorporated by reference. The amplified target nucleic acids are then detected by hybridization methods as described above.

In another aspect, the method of the present invention may be used to prevent the inhibition of other enzymes commonly used in industry, medical clinics, research laboratories and commercial formulations. For example, the method of the present invention may be used to prevent the inhibition of a restriction  
20 enzyme digestion of a nucleic acid in a sample containing an anionic detergent without the need to remove the anionic detergent from solution first.

While the foregoing disclosure describes the problem solved by the present invention and its general means of solution, it will be understood that the foregoing disclosure does not in any way limit the present invention or its application, which are defined solely by the claims which follow this disclosure.

## 25 SUMMARY OF THE INVENTION

The present invention features a method for conducting a enzymatic reaction in a solution containing an ionic detergent, such as sodium dodecyl sulphate (SDS) or lithium lauryl sulphate (LLS), at a concentration  
30 which is normally sufficient to inhibit the activity of the enzyme.

In one embodiment, the present invention relates to a method for preventing the inhibition of at least one enzyme in a nucleic acid target amplification reaction. The method concerns the addition to a solution containing an anionic detergent (preferably SDS or LLS) and nucleic acids to be analyzed; of, generally, two or more different oligonucleotide primers; one primer having a sequence sufficiently complementary and the  
35 other a sequence sufficiently analogous to a target nucleic acid sequence; four different nucleotides; necessary salts and cofactors such as  $MgCl_2$  or NaCl; and an amount, preferably between 8-20% (v/v), of a non-ionic detergent, preferably polyoxyethylene (20) sorbitan monoalkylates (the Tween series of detergents) or polyoxyethylene p-t-octyl phenol derivatives (e.g., Triton X-100 and Triton X-102). These constituents are mixed, and a nucleic acid polymerase, preferably a heat-stable DNA polymerase such as  
40 the DNA polymerase from the bacterium *Thermus aquaticus* (Taq DNA polymerase), is added to the reaction mixture and the mixture is subjected to a nucleic acid amplification procedure, preferably the polymerase chain reaction (PCR). In another preferred embodiment two enzymes are used in the amplification reaction: a reverse transcriptase having RNase H activity, such as MMLV RT, and a RNA polymerase, such as T7 polymerase.

45 In another preferred embodiment, the method of the present invention is used to prevent inhibition of a restriction enzyme in the presence of sodium dodecyl sulfate (SDS). Plasmid pUC19 (Bethesda Research Laboratories, Gaithersburg, MD) was digested with restriction endonuclease Eco R1 at the single Eco R1 site. Following linearization, the plasmid DNA was radiolabeled with  $[\alpha\text{-}^{32}\text{P}]$  dATP using the Klenow fragment of *E. coli* DNA polymerase I. The radiolabeled probe was precipitated and resuspended in a solution  
50 containing differing amounts of SDS and Tween-20. The DNA was then given an excess of restriction endonuclease Pvu II, and incubated for one hour at 37°C. The resulting samples were analyzed on a 7% polyacrylamide gel under non-denaturing conditions.

Although hydrophobic relationships probably play an important role in the deinhibition of the enzymatic reactions by removing enzyme-inhibiting ionic detergents from solution, micelle formation alone may not be  
55 the only determinant as to the suitability of a particular non-ionic detergent in the method described herein. Thus, while the formation of mixed micelles is thought by the Applicants to be important in the practice of this invention, the Applicants remain uncertain as to the precise mechanism which allows enzyme activity to be preserved in the presence of ionic detergent.

It is an object of the present invention to provide a method for conducting a target amplification reaction in the presence of normally inhibiting or denaturing amounts of an ionic detergent such as LLS or SDS.

It is also an object of the present invention to provide a method for conducting a qualitative and/or a quantitative analysis of a biological or clinical sample containing nucleic acids and an ionic detergent such as LLS or SDS, wherein such an analysis involves at least one enzyme-mediated reaction, such as the amplification of a specific nucleic acid sequence or a restriction digest, without the need to separate the nucleic acid analyte from the ionic detergent before commencing the reaction.

It is another object of the present invention to provide a method for preventing the inhibition of a nucleic acid amplification reaction in a test sample wherein the sample includes: 1) nucleic acids to be tested for the presence or absence of a specific nucleic acid sequence; and 2) from about 0.1-1.5% of an ionic detergent, preferably about 0.3-0.7% SDS or LLS, and where the enzyme or enzymes to be employed in the nucleic acid amplification reaction are preferably Taq DNA polymerase, T7 DNA polymerase, and/or retroviral RNA-directed DNA polymerase (reverse transcriptase or RT); most preferably Taq DNA polymerase. The method comprises the steps of adding non-ionic detergent, preferably between about 8-20% of polyoxyethylene (20) sorbitan monolaurate (Tween-20) or polyoxyethylene (9) p-t-octyl phenol (Triton X-100) to the nucleic acids and ionic detergent, mixing the solution together vigorously to form heterogeneous detergent micelles, adding an effective amount of a nucleic acid polymerase, preferably between about 2-8 units of Taq DNA polymerase and subjecting the reaction mixture to a nucleic acid amplification procedure, such as PCR.

It is further an object of this invention to provide a kit for either the qualitative analysis or quantitative analysis, or both, of biological samples containing nucleic acids or microorganisms which contain nucleic acids. When the sample contains microorganisms, such a kit should provide a lysis, permeabilization, or sample transport reagent including between about 0.1%-1.5% of an ionic detergent, preferably SDS or LLS, to inhibit protease and nuclease activity and to assist in dissolution of the cell wall and membrane. In such a reagent, the liberated nucleic acids are protected from nuclease degradation by the ionic detergent. The kit should also provide a second reagent containing: 1) generally two or more nucleic acid primers having nucleic acid sequences sufficiently complementary to that of a target nucleic acid sequence to form, under hybridization conditions, a hydrogen-bonded region with either or both of the complementary nucleic acid strands having such a target sequence; 2) from about 0.5-10 mM  $MgCl_2$  or  $MnCl_2$ ; 3) an effective amount of a disulfide cleaving agent, preferably from about 0.5-10 mM dithiothreitol (DTT); and 4) from 8 to 20% (v/v) of a non-ionic detergent, preferably polyoxyethylene (20) sorbitan monolaurate (Tween-20) or polyoxyethylene (20) sorbitan monooleate (Tween-80). An aliquot of the sample solution containing the liberated nucleic acids in the lysis, permeabilization or transport medium is added to the second solution, and mixed vigorously. From about 2-8 units of at least one nucleic acid polymerase, preferably Taq DNA polymerase, is then added to the reaction mixture and the mixture is treated according to the normal procedure for the polymerase chain reaction. Following this, the sample is tested for the presence or absence of at least one specific nucleic acid sequence using nucleic acid hybridization methods and a labeled nucleic acid probe.

It is yet another object of the present invention to provide a method for the amplification of nucleic acids in the presence of an ionic detergent, preferably about 0.3 to 0.7% LLS. In this method, the final composition of the starting solution includes the following: 1) about 50 mM Tris-HCl (pH 7.6), 17.5 mM  $MgCl_2$ , 25 mM KCl and 2 mM spermidine, 2) about 25 mM each of four different ribonucleotide triphosphates (A,U,G and C) and four different deoxyribonucleotide triphosphates (A,T,G and C), 3) about 1 mM DTT, 4) about 20 picomoles of, generally, two or more single-stranded nucleic acid primers which have nucleic acid sequences sufficiently complementary to that of a target nucleic acid sequence to form a double-stranded, hydrogen-bonded region with either a positive or a negative sense nucleic acid strand having such a target nucleic acid sequence, or both, under hybridization conditions, 5) between about  $6 \times 10^{-3}$  to  $6 \times 10^{-9}$  picomoles of a nucleic acid having at least one copy of a specific target nucleic acid sequence.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The claimed method and kit feature a series of steps for conducting a target amplification reaction in the presence of an ionic detergent, as well as a combination of reagents for accomplishing such steps.

In one of its preferred embodiments the present invention provides a method and means for conducting a nucleic acid amplification reaction when the sample to be analyzed contains ionic detergents. The method involves adding a quantity of a non-ionic detergent to the sample solution and mixing the ionic and the non-ionic detergents together vigorously before the addition of the enzyme or enzymes and initiation of the amplification reaction.

While the Applicants used purified nucleic acids in some of their embodiments, any source of nucleic acids, either purified or unpurified can be used so long as it contains or is suspected of containing the target nucleic acid sequence. This sequence may be present in DNA or RNA, which may be single-stranded or double-stranded. A mixture of these nucleic acids may be used, as may the nucleic acids from a previous amplification reaction. This invention is therefore a generally useful method for overcoming the inhibitory effects of a detergent in a sample containing nucleic acids to be analyzed.

#### Materials

The non-ionic detergents Tween-20, Tween-40, Tween-80, Triton X-100 and Triton X-102 were purchased from the Sigma Chemical Co., St. Louis, Mo.

The nucleic acid primers were synthesized by use of standard phosphoramidite chemistry; various such methods are well known in the art, see e.g., Carruthers *et al.*, 154 *Methods in Enzymology* 287 (1987); Bhatt, U.S. Serial No. 07/319,570 (filed 3/6/89), and Klem *et al.*, PCT WO92/07864, which are hereby incorporated by reference as part of this disclosure. Applicants prepare the probes using a Model 380A DNA synthesizer (Applied Biosystems, Inc., Foster City, CA).

#### Detection Method

The amplification products were quantified using a detectable chemiluminescent acridinium ester-labeled nucleic acid probe that hybridizes to the target sequence. In particular, a double-stranded hybrid molecule is detected by contacting the amplified sample with the labeled probe under specified hybridization conditions, selectively hydrolyzing the acridinium ester bound to unhybridized probe, and measuring the chemiluminescence of the remaining acridinium ester (i.e. that associated with double-stranded nucleic acid regions) in a luminometer. See e.g., Arnold *et al.*, PCT US88/02746 and Nelson *et al.*, in *Nonisotopic DNA Probe Techniques* 275 (Academic Press, San Diego 1992), which are hereby incorporated by reference.

The following examples do not necessarily represent optimal conditions for the use of the present invention; they are intended to be exemplary only and represent currently preferred embodiments of the present invention. These examples are not intended to be limiting as to the scope of possible embodiments of the claimed method, such embodiments being immediately apparent to those of skill in the art upon exposure to the present disclosure.

#### Example 1

This example demonstrates the effectiveness of using the present method in a transcription-based amplification system, see Kacian & Fultz, *supra*. The target nucleic acid for this example was a solution of total ribosomal RNA purified from *Ureaplasma urealyticum* (0.6 picomoles/ml) in a buffer containing 30 mM sodium phosphate pH (6.7), 1.0 mM disodium EDTA (ethylenediaminetetracetic acid disodium), 1 mM EGTA (ethylene glycol-bis ( $\beta$ -aminoethyl ether) N,N,N',N'-tetracetic acid), and 110 mM (3.0% w/v) LLS. Serial dilutions were made in the same buffer.

A solution of 25% (v/v) Triton X-102 in deionized water was prepared. Reaction volumes were 100  $\mu$ l, and reaction was conducted in microcentrifuge tubes. Each tube was given 10  $\mu$ l of a buffer containing 500 mM Tris-HCl (pH 7.6), 175 mM MgCl<sub>2</sub>, 250 mM KCL, and 20 mM spermidine, 0.5  $\mu$ l of 1 M DTT, 10  $\mu$ l of a solution of ribonucleotides containing 25 mM rCTP and rUTP and 65 mM rATP and rGTP, 2  $\mu$ l of a solution containing 100 mM each of dATP, dTTP, dCTP, and dGTP, 8.6  $\mu$ l of a solution of 30 pM of the first primer (81(-); SEQ ID NO: 1), 3.4  $\mu$ l of a solution of 30 pM of the second primer (uur C 1(+); SEQ ID NO: 2), 10  $\mu$ l of undiluted, 1:10 diluted, or 1:100 diluted target rRNA prepared as described above, varying amounts of the 25% (v/v) Triton X-102 solution, and water to a volume of 93.6  $\mu$ l. The sequences of these primers are as follows:

SEQ ID NO 1:

5

55

AATTTAATACGACTCACTATAGGGAGAGCGTAGCGATGACCTATTTTACTTGC-3'



SEQ ID NO 2:

5'-TG TAGT GATCATATCAGAGTGG-3'

Each reaction tube was mixed vigorously using a vortex mixer, and then the samples were heated to 95 °C for 2 minutes and cooled to room temperature. Moloney Murine Leukemia Virus RNA-directed DNA polymerase (MMLV reverse transcriptase, 300 units) and 400 units of T7 RNA polymerase were separately added to each tube in a total volume of 6.44 µl. The samples were then incubated at 37 °C for 4 hours. Tubes were cooled to 4 °C awaiting the detection step.

Quantification of amplified target sequences was performed according to the HPA chemiluminescence method referred to above and incorporated by reference as part of this disclosure. See Arnold et al., *supra*. One of two labeled probes specific to the target sequence were used in the hybridization assay. Their sequences are as follows:

SEQ ID NO:3

5'-GTGATCATATCAGAGTGGAATACCTGTTCCCATCC-3'

SEQ ID NO:4

5'-GCTTGTGTCTTCAGTTCGTGAGATCTCGGC-3'

### Example 2

This example demonstrates the ability of a polyoxyethylene(9) p-t-octyl phenol derivative (Triton X-102) to act as the non-polar detergent in the method of the present invention. The concentration of LLS was 0.3% (w/v). Triton X-102 concentrations were 9%, 10%, and 11% (v/v). Duplicate experiments were run for each condition, and duplicate HPA detections made for each experiment; the resulting values were averaged. Amount of amplification is proportional to the amount of label detected; this quantity is given in RLU or relative light units. Values appearing in parentheses were control experiments. The results of the amplification experiments are shown below in Table 1.

TABLE 1

Amplification Reaction in Presence of 0.3% (w/v) LLS and Triton X-102 [Average Relative Light Units (RLU)]			
	Input Target RNA (picomoles)		
	$6 \times 10^{-3}$	$6 \times 10^{-4}$	$6 \times 10^{-5}$
No LLS, no Triton X-102	( $5.2 \times 10^7$ )		
No Target RNA	(100)		
10% Triton X-102; no LLS	( $1.7 \times 10^8$ )		
9% Triton X-102	24,000	770	530
10% Triton X-102	72,000	280	100
11% Triton X-102	78,000	87,000	87,000

### Example 3

The following experiment demonstrates the sensitivity of nucleic acid amplification according to the presently claimed method. The experiment was conducted as in Example 1, with the following exceptions. Tween-20 was substituted for Triton X-102, and the amounts of added target RNA were  $6 \times 10^{-3}$ ,  $6 \times 10^{-5}$ ,  $6 \times 10^{-7}$  and  $6 \times 10^{-9}$  picomoles. Final concentrations of Tween-20 were 13, 15 and 20% (v/v). The results are shown in Table 2.

TABLE 2

Sensitivity of Amplification Reaction in Presence of 0.3% (w/v) LLS and 20% (v/v) Tween-20 [Average Relative Light Units (RLU)]				
	Input Target RNA (picomoles)			
	$6 \times 10^{-3}$	$6 \times 10^{-4}$	$6 \times 10^{-5}$	$6 \times 10^{-6}$
No LLS, no Tween-20		$2.3 \times 10^6$	$2.3 \times 10^6$	$2.2 \times 10^6$
LLS, Tween; no target	(6,300)			
13% Tween-20	$2.4 \times 10^6$	$1.9 \times 10^6$	$1.3 \times 10^6$	$1.2 \times 10^6$
15% Tween-20	$2.3 \times 10^6$	$2.2 \times 10^6$	$1.2 \times 10^6$	940,000
20% Tween-20	$2.3 \times 10^6$	$1.6 \times 10^6$	310,000	320,000

Example 4

This example demonstrates the effectiveness of the present method for use with nucleic acid amplification employing the polymerase chain reaction (PCR). For this experiment the target nucleic acid was a restriction fragment having a known DNA sequence obtained from a plasmid containing the E6 gene of Human Papilloma Virus. The two oligonucleotide primers (SEQ ID NO:4 AND SEQ ID NO: 5) were designed to be complementary to opposite strands of the double-stranded target nucleic acid. The sequences of these primers are as follows:

SEQ ID NO: 5

5'-GCAATGTAGGTGTATCTCC-3'

SEQ ID NO: 6

5'-TATGCACAGAGCTGCA-3'

The final reaction volume for each sample was 100  $\mu$ l. In this experiment, 2 drops of mineral oil were added to each tube, followed by a precalculated amount of water. One microliter of target DNA in 100 mM Tris-HCl pH 7.6, 10 mM EDTA ( $1 \times 10^4$  copies of the target sequence) was then added. A buffer containing 30 mM sodium phosphate pH 6.7, 1.0 mM disodium EDTA, 1 mM EGTA, and 110 mM (3.0% w/v) LLS was added to some tubes to give a final LLS concentration of 0.3%. Differing volumes of a 50% (v/v) solution of Tween-20 was also added to some tubes to give a reaction concentration of 10%, 12%, 14%, 16%, 18%, 20%, 22%, or 24%. Following the addition of detergent the samples were mixed on a vortex mixer.

A PCR premix was made separately, containing 10  $\mu$ l of 10X PCR buffer, 20 nanomoles of each deoxyribonucleotide, 100 picomoles of each nucleic acid primer and 2.5 units of *Taq* DNA polymerase for each reaction tube. The sample tubes were placed on ice, and the PCR premix was added to each sample and mixed. Each sample was heated to 94 °C for 3 minutes, then taken through 35 thermocycles comprising the following: incubation at 51 °C for 30 seconds, followed by incubation at 72 °C for 2 minutes, and finally at 94 °C for 1 minute. Following the final 94 °C incubation, the samples were incubated at 72 °C for 5 minutes then stored at 4 °C overnight for detection.

Detection was carried out as in Example 1 using a probe complementary to the target nucleic acid sequence. The sequence of the probe is as follows:

SEQ ID NO: 7

5'-GAACAGCAATACAACAAACCGTTGTGTG-3'

The results are shown in Table 3.

TABLE 3

PCR Amplification in the Presence of 0.3% (w/v) LLS	
	Average RLU
No LLS, no Tween	290,000
10% Tween-20	310,000
12% Tween-20	250,000
14% Tween-20	360,000
16% Tween-20	160,000
18% Tween-20	320,000
20% Tween-20	180,000
22% Tween-20	120,000
24% Tween-20	140,000

Example 5

This experiment shows the effectiveness of Tween-20 to prevent the inhibition of Taq DNA polymerase-mediated PCR at different concentrations of LLS and Tween-20. The experiment was conducted as in Example 3, except as follows: LLS concentrations were made 0%, 0.3%, 0.5%, and 0.7% (w/v). Tween-20 concentrations were variously 0%, 10%, 14%, 18%, and 20% (v/v). The results are shown in Table 4.

TABLE 4

PCR Amplification in Presence of Various Concentrations of LLS and Tween-20 [Average RLU]				
	0% LLS	0.3% LLS	0.5% LLS	0.7% LLS
0% Tween-20	410,000	14	25	45
10% Tween-20	120,000	11,000	290	300
14% Tween-20	170,000	170,000	64,000	280
18% Tween-20	230,000	210,000	77,000	340
20% Tween-20	170,000	190,000	42,000	310

Example 6

This experiment was performed as is Example 4, except Tween-40 (polyoxyethylene (20) sorbitan monopalmitate) was substituted for Tween-20. The results are shown in Table 5.

TABLE 5

PCR Amplification in Presence of Various Concentrations of LLS and Tween-40 [Average RLU]				
	0% LLS	0.3% LLS	0.5% LLS	0.7% LLS
0% Tween-40	2,500,000	48,000	4,900	5,100
10% Tween-40	160,000	260,000	5,400	5,100
14% Tween-40	180,000	110,000	65,000	5,200
18% Tween-40	150,000	82,000	52,000	17,000
20% Tween-40	77,000	49,000	32,000	6,800

Example 7

This experiment was conducted as in Example 4, except Tween-80 (polyoxyethylene (20) sorbitan monooleate) was substituted for Tween-20. Experimental compositions which were not tested are indicated "ND". The results are shown in Table 6.

TABLE 6

PCR Amplification in Presence of Various Concentrations of LLS and Tween-80 [Average RLU]				
	0% LLS	0.3% LLS	0.5% LLS	0.7% LLS
0% Tween-80	2,500,000	ND	0.0	ND
10% Tween-80	2,200,000	61,000	1,100,000	230,000
14% Tween-80	690,000	260,000	130,000	0.0
18% Tween-80	900,000	750,000	140,000	94
20% Tween-80	330,000	460,000	39,000	260,000

Example 8

This experiment demonstrates the effectiveness of the present method for use with biological samples suspected of containing nucleic acids or nucleic acid-containing microorganisms. The sample was a pool of endo-cervical swab specimens in a buffer containing 30 mM sodium phosphate (pH 6.8), 1.0 mM disodium EDTA (ethylenediaminetetracetic acid disodium), 1 mM EGTA (ethylene glycol-bis ( $\beta$ -aminoethyl ether) N,N,N',N'-tetracetic acid), and 110 mM (3.0% w/v) LLS which tested positive for *Chlamydia trachomatis* in a previous direct nucleic acid hybridization assay without amplification.

The samples were treated with 40  $\mu$ l of a Proteinase K solution (0.1 unit/ $\mu$ l) for each 800  $\mu$ l of sample, and incubated at 60 °C for 20 minutes. Tween-80 (polyoxyethylene (20) sorbitan monooleate) was added to a sample dilution buffer containing 40 mM Tris-HCl (pH 8.0), 10 mM N-acetyl-L-cysteine (NALC), and 2 mM EDTA. Amounts of Tween-80 were added to this buffer in order to make the final Tween concentration during the amplification 6.4%, 6.8%, 7.2%, 7.6%, 8%, 10% and 12%. Ten microliters of the specimen sample was combined with 40  $\mu$ l of each concentration of non-ionic detergent in dilution buffer and mixed vigorously using a vortex mixer, then stored for either 3-4 days at 4 °C.

25  $\mu$ l of an amplification buffer containing 20% (w/v) polyvinylpyrrolidone, 16 mM each of rCTP, rATP and rUTP and 20 mM rGTP, 6 mM deoxyribonucleotides, 160 mM Tris pH 7.5, 92 mM MgCl<sub>2</sub>, 92 mM KCl, 3.0 picomoles of a first primer (T7 AproCtRB(-)1519; SEQ ID NO: 7) and 25.0 picomoles of a second primer (CtRB(+)1428b; SEQ ID NO: 8) was pipetted into a separate tube for each experiment, and 200  $\mu$ l of mineral oil was layered on top of each tube. One of the oligonucleotide primers had a nucleic acid sequence analogous to for a region of *Chlamydia trachomatis* ribosomal RNA; the other oligonucleotide primer had a nucleic acid sequence complementary to a region of *Chlamydia trachomatis* ribosomal RNA.

The sequences of these primers are as follows:

SEQ ID NO: 8:

5'-AATTTAATACGACTCACTATAGGGAGACCCGAAGATTCCTTGATCGC-3'

SEQ ID NO: 9:

5'-CGGAGTAAGTTAAGCACGCGGACGATTGGAAGA-3'

The 50  $\mu$ l containing each sample and detergent combination was pipetted through the layered mineral oil into the amplification buffer. Each tube was heated at 95°C for 5 minutes in a heating block, then transferred to a 42°C heating block for another 5 minutes before the addition of enzyme. Twenty-five microliters of an enzyme solution containing 900 units Moloney Murine Leukemia Virus reverse transcriptase (MMLV RT) and 400 units T7 RNA polymerase was added to each tube and mixed into solution. Each tube was then incubated for 42°C for 1 hour, then treated with 20  $\mu$ l of a solution containing 50 units of essentially RNase-free DNase and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) in ethanol. Samples were incubated for another 10 minutes at 42°C and HPA detection was carried out as described above using a labeled oligonucleotide probe specific for the target *Chlamydia trachomatis* ribosomal RNA. The sequence of the probe is as follows:

SEQ ID NO: 10

5'-AGAGTCCGTAGAGCGATGAGAACG-3'

Positive controls are indicated in parentheses, and were samples in which the indicated amount of RNA, not contained in a biological sample and in a final concentration of 0.3% LLS, was subjected to the amplification procedure. Each experimental sample was run in duplicate. The results are indicated in the Table 7.

TABLE 7

Amplification of Nucleic Acids in Biological Samples Stored in Presence of Heterogeneous Micelles [Average RLU]	
	<i>C. trachomatis</i> positive pool in 0.3% LLS
No target	1600
0.5 fg <i>C. trachomatis</i> rRNA	2,000,000
5.0 fg <i>C. trachomatis</i> rRNA	1,300,000
Sample in 6.4% Tween-80	150,000
Sample in 6.8% Tween-80	2,400,000
Sample in 7.2% Tween-80	2,300,000
Sample in 7.6% Tween-80	2,800,000
Sample in 8.0% Tween-80	2,900,000
Sample in 10% Tween-80	3,000,000
Sample in 12% Tween-80	2,900,000

#### Example 9

This example illustrates the effectiveness of the method of the present invention to prevent the inhibition of enzyme-mediated reactions by ionic detergents in systems other than nucleic acid amplification reactions. Five micrograms of plasmid pUC19 (Bethesda Research Laboratories, Gaithersburg, MD) were digested with restriction endonuclease Eco R1 at its sole Eco R1 site in a reaction mixture containing 5  $\mu$ l of 10X High Salt buffer (10 mM NaCl, 50 mM Tris (pH 7.5), 10 mM MgCl<sub>2</sub> and 1 mM DTT), 48 units of Eco R1 (48 units/ $\mu$ l) in a final reaction volume of 50  $\mu$ l. The reaction mixture was incubated at 37°C for 1 hour. The sample was then precipitated with a solution containing 300  $\mu$ l of ethanol, 5  $\mu$ l of 3 M sodium acetate, and 1  $\mu$ l of glycogen and redissolved in 16  $\mu$ l water.

Following linearization of the circular plasmid, the plasmid was radiolabeled with [ $\alpha$ ]-<sup>32</sup>P dATP using the Klenow fragment of *E. coli* DNA polymerase I as follows. The dissolved DNA was given 2  $\mu$ l of High Salt buffer, 1  $\mu$ l of [ $\alpha$ ]-<sup>32</sup>P dATP, and 1  $\mu$ l of the Klenow fragment (5 units/ $\mu$ l) (Stratagene, San Diego, CA). The

reaction mixture was incubated for 20 minutes at room temperature, then precipitated by adding 2  $\mu$ l of 3 M sodium acetate and 200  $\mu$ l ethanol, and pelleted in a microcentrifuge. The DNA was redissolved in 100  $\mu$ l of water.

To test whether the addition of Tween-20 to a solution containing SDS and the radiolabeled DNA would prevent inhibition of digestion of the linearized DNA by restriction endonuclease Pvu II, different reaction mixtures were made up with varying amounts of the two detergents, as shown below. Each reaction was in a total volume of 50  $\mu$ l. SDS and Tween-20 were added to the reaction mixture prior to the addition of the radiolabeled DNA. 5 microliters of 10X REACT buffer (500 mM Tris-HCl (pH 7.4), 60 mM MgCl<sub>2</sub>, 500 mM NaCl, and 500 mM KCl) were also added to the reaction mixture. Ten units of Pvu II (10 units/ $\mu$ l) were then added to each tube, along with 1  $\mu$ l (50 ng) of the radiolabeled DNA. The reaction mixture was incubated at 37 °C for 1 hour. Five microliters of each digestion mixture was added to 10  $\mu$ l of non-denaturing gel loading buffer and 5  $\mu$ l of water, and an aliquot of each sample was analyzed on a 7% polyacrylamide non-denaturing gel. Molecular weight markers were radiolabeled  $\phi$ X174 DNA digested with Hinf I. The gel was run until the running dye had reached the bottom of the gel, and the gel was used to expose X-ray film for either 4 hours or overnight. The X-ray film was exposed (Figure 1), and the results were analyzed.

PERCENT SDS IN EACH SAMPLE		
Gel Lanes	Percent SDS	Microliters 2% SDS Solution
1-5	0	0
6-10	0.01	2.5 (10X dilution)
11-15	0.05	1.25
16-20	0.1	2.5
21-25	0.5	12.5

PERCENT TWEEN-20 IN EACH SAMPLE		
Gel Lane	Percent Tween-20	Microliters 50% (v/v) Tween-20
1,6,11,16,21	0	0
2,7,12,17,22	10	10
3,8,13,18,23	14	14
4,9,14,19,24	18	18
5,10,15,20,25	20	20

The gel lanes are numbered from left to right. The two leftmost lanes are the molecular weight markers, with their sizes (in number of bases) indicated next to the bands in the overnight exposure, followed by a negative control of Pvu II undigested, Eco R1 linearized pUC19 DNA. The lane to the far right also contains the molecular weight markers.

The Eco R1 site in the 2686 bp pUC19 plasmid is at map position 396. The Pvu II sites in pUC19 are at map positions 306 and 628. Thus, a complete digestion of the pUC19 DNA would yield expected fragments of 90, 232, and 2364 bp in length.

The results, shown in Figure 1, indicate that Pvu II digestion is inhibited by concentrations of SDS as low as 0.01% (w/v). However, the addition of 10-20% Tween-20 to the reaction mixture before addition of the enzyme allows the restriction digestion to occur.

The foregoing examples demonstrate currently preferred embodiments of the present invention. However, it will be immediately appreciated by those skilled in the art in light of this disclosure that other combinations of enzymes, ionic detergents and non-ionic detergents may be used in the method of the present invention. Other such combinations may be screened for use in the present invention without undue experimentation using the methods disclosed herein in conjunction with standard enzyme assay procedures.

INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

5

(A) LENGTH: 53  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

10

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

AATTTAATAC GACTCACTAT AGGGAGAGCG TAGCGATGAC CTATTTTACT TGC 53

15

INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

20

(A) LENGTH: 22  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

25

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

TGTAGTGATC ATATCAGAGT GG

22

INFORMATION FOR SEQ ID NO: 3:

30

(i) SEQUENCE CHARACTERISTICS:

35

(A) LENGTH: 36  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

40

GTGATCATAT CAGAGTGGAA ATACCTGTTC CCATCC

36

INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

45

(A) LENGTH: 30

50

55

(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

5 GCTTGTGTCT TCAGTTCGTG AGATCTCGGC 30

INFORMATION FOR SEQ ID NO: 5:

10 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
15 (D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 5

GCAATGTAGG TGTATCTCC 19

20 INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16  
25 (B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 6

30 TATGCACAGA GCTGCA 16

INFORMATION FOR SEQ ID NO: 7:

35 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
40 (D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GAACAGCAAT ACAACAAACC GTTGTGTG 28

45 INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 49  
50 (B) TYPE: nucleic acid

55



(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

5 (ii) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

AATTTAATAC GACTCACTAT AGGGAGACCC GAAGATTCCC CTTGATCGC 49

INFORMATION FOR SEQ ID NO: 9:

10

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33  
(B) TYPE: nucleic acid  
15 (C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

20 CGGAGTAAGT TAAGCACGCG GACGATTGGA AGA 33

INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

25

(A) LENGTH: 24  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
30 (D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

AGAGTCCGTA GAGCGATGAG AACG 24

35

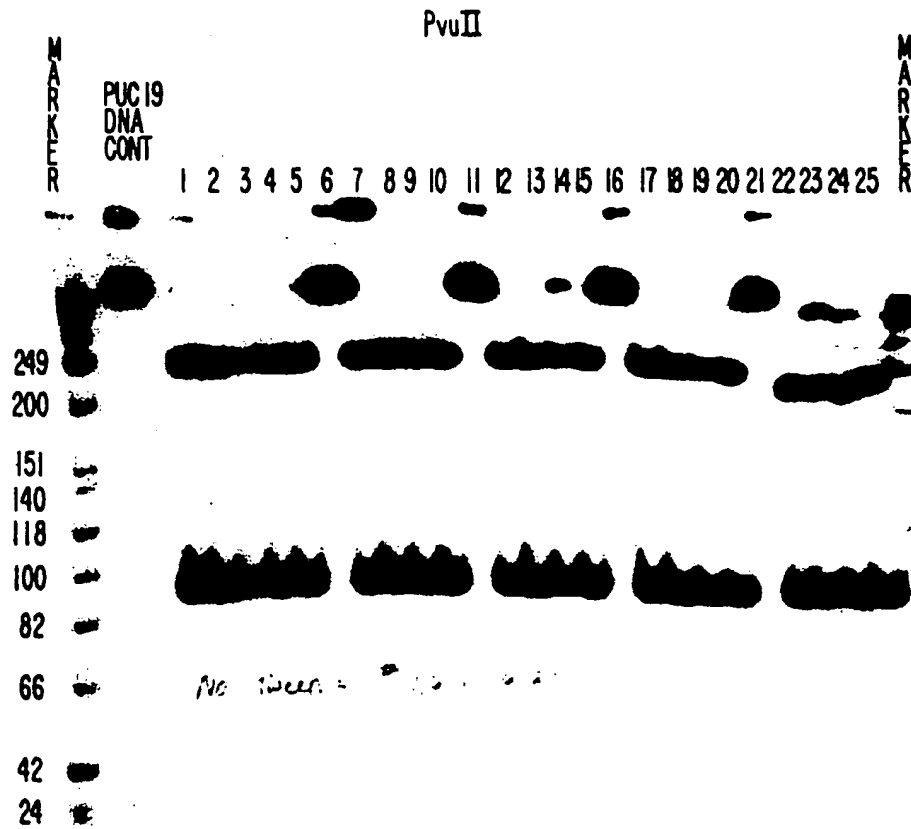
# Claims

1. A method for enhancing enzymatic activity in a liquid containing an ionic detergent and at least one enzyme substrate, comprising the steps of:  
40 a. adding a non-ionic detergent to the liquid in an amount sufficient to suppress inhibition of at least one enzyme activity by said ionic detergent in said liquid;  
b. mixing together the liquid comprising the ionic detergent, the non-ionic detergent and the enzyme substrate;  
45 c. adding at least one enzyme having said activity to the mixture; and  
d. incubating the mixture under reaction conditions favorable to said enzymatic activity.
2. The method of claim 1 wherein the liquid comprises a biological sample.
- 50 3. The method of claim 1 or 2 wherein the enzyme substrate comprises a nucleic acid.
4. The method of claim 1, 2 or 3 wherein the enzyme is selected from the group consisting of a DNA-directed DNA polymerase, an RNA-directed DNA polymerase, a DNA-directed RNA polymerase, a RNA-hydrolyzing enzyme, a restriction endonuclease and a protease.
- 55 5. The method of claim 4 wherein the enzyme is selected from the group consisting of retrovirus-derived enzymes having RNA-directed DNA polymerase activity, DNA polymerases derived from Thermus aquaticus, DNA polymerases derived from Bacillus sterothermophilus, restriction endonucleases and T7

RNA polymerase.

6. The method of any of the claims 1-5 wherein the ionic detergent comprises a composition selected from the group consisting of water-soluble lauryl sulfate salts.
7. The method of claim 6 wherein the non-ionic detergent is selected from the group consisting of lithium lauryl sulfate and sodium dodecyl sulfate.
8. The method of any of the claims 1-7 wherein the final concentration of the ionic detergent is between about 0.1-0.7%.
9. The method of any of the claims 1-8 wherein the non-ionic detergent is selected from the group consisting of polyoxyethylene (20) sorbitan mono-alkylate derivatives.
10. The method of claim 9 wherein the non-ionic detergent comprises a composition selected from the group consisting of polyoxyethylene (20) sorbitan monolaurate, polyoxyethylene (20) sorbitan monopalmitate, polyoxyethylene (20) sorbitan monostearate and polyoxyethylene (20) sorbitan monooleate.
11. The method of any of the claims 1-10, wherein the final concentration of the non-ionic detergent is between about 6-20%.
12. A method for conducting nucleic acid amplification reactions of biological samples containing nucleic acids in the presence of an ionic detergent comprising the steps of:
  - a. contacting the biological sample with a first liquid comprising an ionic detergent,
  - b. mixing the first liquid with a second liquid comprising a non-ionic detergent, thereby forming a third liquid,
  - c. adding to the third liquid at least one nucleic acid primer having a nucleic acid sequence sufficiently complementary to a target nucleic acid sequence, nucleoside triphosphates, necessary cofactors or salts, and at least one enzyme having nucleic acid polymerase activity,
  - d. incubating the mixture under conditions sufficient to cause amplification of at least one target nucleic acid sequence, and
  - e. detecting the amplified target sequence.
13. The method of claim 12 wherein the ionic detergent comprises a composition selected from the group consisting of water-soluble lauryl sulfate salts.
14. The method of claim 13 wherein the ionic detergent is selected from the group consisting of lithium lauryl sulfate and sodium dodecyl sulfate.
15. The method of any of the claims 12-14 wherein the final concentration of the ionic detergent is between about 0.1-0.7%.
16. The method of any of the claims 12-15 wherein the non-ionic detergent comprises a composition selected from the group consisting of polyoxyethylene (20) sorbitan monoalkylate derivatives.
17. The method of claim 16 wherein the non-ionic detergent comprises a composition selected from the group consisting of polyoxyethylene (20) sorbitan monolaurate, polyoxyethylene (20) sorbitan monooleate, polyoxyethylene (20) sorbitan monopalmitate, and polyoxyethylene (20) sorbitan monostearate.
18. The method of any of the claims 12-17 wherein the non-ionic detergent has a final concentration between about 6-20%.
19. The method of any of the claims 12-18 wherein the target nucleic acid sequence comprises at least one sequence specific to ribosomal RNA or ribosomal DNA.
20. The method of claim 19 wherein the ribosomal RNA sequences are specific for microorganisms selected from the group consisting of Chlamydia trachomatis, Ureaplasma urealyticum, and human cells infected with Human Papilloma Virus.

21. The method of any of the claims 12-20 wherein the first liquid further comprises a non-ionic detergent and a metal chelating agent.
22. The method of any of the claims 12-21, further comprising the step of:
  - a. adding a RNase-inhibiting agent to the third liquid, and
  - b. optionally inactivating the RNase-inhibiting agent prior to the addition of the nucleic acid polymerase.
23. The method of claim 22 wherein the RNase-inhibiting agent comprises a protease or proteinase K.
24. The method of claim 22 or 23 wherein the step of inactivating the RNase-inhibiting agent comprises:
  - a. heating the reaction mixture to between about 90-100 °c for between about 1-5 minutes.
25. A kit for amplifying a target nucleic acid sequence in a biological sample, said kit comprising:
  - a. a first reagent for collecting and transporting the sample containing an ionic detergent,
  - b. a second reagent containing a non-ionic detergent for mixing with the first reagent,
  - c. an amplification reagent containing at least one nucleic acid primer sufficiently complementary to the target nucleic acid sequence, nucleoside triphosphates, cofactors and salts,
  - d. at least one enzyme having nucleic acid polymerase activity for initiating the nucleic acid amplification reaction.
26. The kit of claim 25 wherein the ionic detergent comprises a composition selected from the group consisting of lauryl sulfate salts.
27. The kit of claim 26 wherein the ionic detergent is selected from the group consisting of lithium lauryl sulfate and sodium dodecyl sulfate.
28. The kit of any of the claims 25-27 wherein the final concentration of the ionic detergent is between about 0.1-0.7%.
29. The kit of any of the claims 25-28 wherein the first reagent further comprises a non-ionic detergent and a metal chelating agent.
30. The kit of any of the claims 25-29 wherein non-ionic detergent is selected from the group consisting of polyoxyethylene (20) sorbitan monoalkylate derivatives.
31. The kit of claim 30 wherein the non-ionic detergent comprises a composition selected from the group consisting of polyoxyethylene (20) sorbitan monolaurate, polyoxyethylene (20) sorbitan monooleate, polyoxyethylene (20) sorbitan monopalmitate, and polyoxyethylene (20) sorbitan monostearate.
32. The kit of any of the claims 25-31 wherein the final concentration of the non-ionic detergent is between about 0.1-0.7%.
33. The kit of any of the claims 25-32 further comprising a RNase-inhibiting agent to be added to the reaction mixture after the formation of heterogeneous detergent micelles.
34. The kit of claim 33 wherein the RNase-inhibiting agent is a protease or proteinase K.
35. The kit of any of the claims 25-34 wherein the target nucleic acid sequence comprises at least one sequence specific to ribosomal RNA or ribosomal DNA.



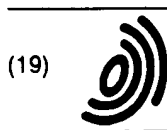


European Patent  
Office

## EUROPEAN SEARCH REPORT

Application Number  
EP 95 10 3520

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
X	US-A-5 175 094 (MALLONEE) 29 December 1992 * column 13, line 4 - line 15 * ---	12,25	C12Q1/00 C12Q1/68 C12P19/34
A	EP-A-0 574 267 (GEN-PROBE INC.) 12 June 1992 * the whole document * ---	1-35	
A	EP-A-0 428 197 (EASTMAN KODAK CO.) 22 May 1991 * the whole document * ---	1-35	
A	EP-A-0 488 243 (SANWA KAGUKU KENYUSHO CO.) 3 June 1992 * claims 1-4 * ---	1,12	
A	WO-A-92 08807 (HRI RESEARCH INC) 29 May 1992 * page 7 - page 11 * ---	1	
T	WO-A-94 26867 (ABBOTT LABS.) 24 November 1994 * page 18, line 1 - page 20, line 7; claims 1-5 * -----	1-35	TECHNICAL FIELDS SEARCHED (Int.Cl.6) C12Q
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 8 June 1995	Examiner Osborne, H
<b>CATEGORY OF CITED DOCUMENTS</b> X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons * : member of the same patent family, corresponding document			



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(54) **Method for suppressing inhibition of enzyme-mediated reactions by ionic detergents**

Verfahren zur Unterdrückung der Hemmung von Enzym-vermittelten Reaktionen durch ionische Detergentien

Procédé pour supprimer l'inhibition de réactions enzymiques par des détergents ioniques

(84) Designated Contracting States:  
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## Description

## TECHNICAL FIELD OF THE INVENTION

5 [0001] The present invention relates to methods for conducting enzymatic reactions in the presence of ionic detergents, for example lithium lauryl sulphate (LLS) and sodium dodecyl sulfate (SDS), which are often present in diagnostic and clinical samples as solubilizing and protein denaturing agents. This invention thereby eliminates the necessity for lengthy and labor-intensive procedures to separate the detergent from an analyte or an enzyme substrate before initiating the desired reaction. The present invention further relates to a method for conducting enzyme-mediated nucleic acid amplification reactions such as the polymerase chain reaction (PCR) or restriction enzyme digests in the presence of ionic detergents such as LLS without the need for a detergent removal step.

## BACKGROUND OF THE INVENTION

15 [0002] This invention concerns enzyme-mediated chemical reactions conducted in vitro, and techniques for preventing their inhibition in the presence of detergents. Detergents are common tools in medical and biological research laboratories, primarily due to their ability to solubilize various proteins, cell wall and membrane components, and other cellular organelles, substructures, and components which are either insoluble or incompletely soluble in aqueous solution alone. Thus, detergents are often components of extraction or lysis buffers, both as lytic agents and as effective inhibitors of undesired enzyme activities such as those contributed by the proteases and nucleases normally present in a crude cell lysate. Additionally, such detergents are often used for the same purposes in the purification of nucleic acids.

20 [0003] Most enzymes used as tools in applied molecular and biological chemistry are quite sensitive to detergents, especially strong ionic detergents such as sodium dodecyl sulphate (SDS) or lithium lauryl sulphate (LLS). Such ionic detergents can bind strongly to proteins, often resulting in irreversible denaturation of the protein. (See American Society for Microbiology, Manual of Methods for General Bacteriology 57-58 (1981)). However, for precisely this reason ionic detergents such as LLS are often an extremely valuable and inexpensive short- to medium-term preservative of nucleic acids in solution. Thus, such agents are useful to assist in accomplishing the first step of a nucleic acid hybridization assay using microorganisms; extraction of the nucleic acids from microbial cells or particles. Ionic detergents help to solubilize the cell wall and cell membrane, and to simultaneously prevent degradation of the nucleic acids by nucleases. (See id.) Moreover, strong ionic detergents such as SDS or LLS are often added to the lysis, permeabilization, or transport media in which clinical specimens are conveyed to the laboratory for analysis.

25 [0004] Often nucleic acids obtained from a biological sample are subsequently subjected to enzymatic manipulation, such as digestion with a restriction endonuclease or an exonuclease specific to DNA or RNA. Additionally, nucleic acids obtained from such samples are often not present in amounts large enough for them to be directly detected and/or quantified by nucleic acid hybridization techniques. Thus, the nucleic acid sequences of interest in such samples must normally be enzymatically amplified to be detected. US Patent No. 5,175,094 discloses a method of preparing Hepatitis B virus (HBV) DNA from serum for polymerase chain reaction (PCR) amplification by adding a buffer containing 0.25% SDS, heat treating the mixture, adding a Taq Polymerase buffer containing 0.5% each of Tween 20® and NP 40, and finally diluting the mixture into a PCR reaction mixture.

30 [0005] WO 92/08807 describes methods of preparing nucleic acid for PCR from whole blood by lysing the blood and treating the lysate with an "interfering reagent" to inactivate polymerase inhibitors in the lysate, such as heme and its related compounds. The "interfering reagent" may be a chemical, biochemical, photochemical, immunological or enzymatic treatment, or combination thereof that inactivates heme-related inhibition.

45 [0006] In biological or clinical samples to be subjected to one or more rounds of nucleic acid amplification or another enzyme-mediated reaction, the detergent must be separated from the nucleic acids in solution before an enzyme can be added to the reaction mixture. Dialysis or ultrafiltration, which usually works well to remove small molecules from a solution will not effectively remove most detergents, probably due both to the size of the micelles formed by the aggregation of the detergent molecules, as well as ionic or hydrophobic binding of the detergent molecules to larger solutes. Moreover, neither dialysis nor ultrafiltration is conveniently adaptable for use in a commercial diagnostic kit.

50 [0007] It would be convenient and cost-effective to perform an enzyme-mediated reaction such as nucleic acid amplification or a restriction digest in the same tube or collection vessel as is used to transport the biological sample to the laboratory for analysis, i.e. in the presence of SDS or LLS. Alternatively, it would be desirable to conduct such a reaction using such a sample as the immediate starting material, rather than having to subject the sample to an additional detergent-removing step. Although SDS can be precipitated with solvents such as acetone, acetone can denature or precipitate some enzymes. Moreover, the desired reaction may be inhibited by traces of acetone or other precipitating agents.

55 [0008] Currently, nucleic acids in a crude sample are generally purified prior to conducting an amplification by means

of a phenol/chloroform extraction and subsequent ethanol precipitation. The method of the present invention takes advantage of both the similarities and the differences between ionic and non-ionic detergents to eliminate the necessity for such a step, thereby allowing enzyme-mediated reactions to be performed using nucleic acids in a biological sample, even when the sample contains an amount of ionic detergent which would normally inhibit the reaction.

5 [0009] The present invention is preferably a method for performing a nucleic acid amplification reaction, such as the polymerase chain reaction (PCR) or a transcription-based amplification system, in the presence of ionic detergents such as sodium dodecyl sulphate (SDS) or lithium lauryl sulphate (LLS). However, this present invention should be capable of preventing the inhibition of other enzymatic reactions, such as restriction digests, endo- and exonuclease digests, and kinase and transferase reactions by ionic detergents as well. Nor does the Applicant contemplate that the  
10 application of the present invention is limited to enzymatic reactions involving nucleic acids. Thus, while the embodiments of the present invention contained herein illustrate the use of the present invention in amplification reactions, such embodiments are meant to be exemplary only, the scope of the present invention being defined solely by the claims with which this specification concludes.

[0010] While not wishing to be bound by theory, Applicants believe that the formation of colloidal aggregates comprising heterogeneous micelles of non-ionic and ionic detergent molecules effectively remove the ionic detergent molecules from solution, thus making them unavailable to bind with or denature the subsequently added enzyme.

[0011] The ability of detergents to enhance or restore the activity of some enzymes has been reported. Saito, M., et al., Action of Arthrobacter ureafaciens Sialidase on Sialoglycolipid Substrates, 254 J. Biol. Chem. 7845-54 (1979). The use of heterogeneous micelles of ionic and non-ionic detergents as a method for the reactivation of detergent-inhibited proteins has also been reported. See Ey, P.L. & Ferber, E., Calf Thymus Alkaline Phosphatase II. Interaction with Detergents, 480 Biochim. Biophys. Acta 163-77 (1977); Berge, R.K., et al., Variations in the Activity of Microsomal Palmitoyl-CoA Hydrolase in Mixed Micelle Solutions of Palmitoyl-CoA and Non-Ionic Detergents of the Triton X Series, 666 Biochim. Biophys. Acta 25-35 (1981); Tandon S., & Horowitz, P.M., Detergent-assisted Refolding of Guanidinium Chloride-denatured Rhodanese, 262 J. Biol. Chem. 4486-91 (1987). Additionally, the formation of heterogeneous micelles of ionic and non-ionic detergents has been reported as a method for removing inhibiting concentrations of non-ionic detergents from a solubilized enzyme preparation. Stralfors, P. et al., Removal of Nonionic Detergent from Proteins Fractionated by Electrofocusing, 533 Biochim. Biophys. Acta 90-97 (1978).  
25

[0012] All of the methods mentioned in the publications listed above involve the activation or reactivation of enzymes in a detergent solution; none of these methods teach or suggest the formation of heterogeneous micelles in a solution containing an enzyme substrate before the addition of active enzyme. Additionally, in all these cases detergents were used to solubilize, purify, or activate the enzyme; in no case was the detergent initially added to solubilize and stabilize the enzyme substrate rather than the enzyme itself.

[0013] The present invention precludes the step of removing ionic detergent from a solution containing substrate before adding an enzyme to promote an enzymatic reaction that would be inhibited by the presence of ionic detergent. By adding a non-ionic detergent to a final concentration of about 6% to 20% (v/v) to the solution containing ionic detergent and substrate, a mixture is produced that suppresses an inhibitory effect of the ionic detergent on enzymatic activity.  
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#### DEFINITIONS

40 [0014] In this application the following terms have the following meanings, unless expressly stated to the contrary herein.

[0015] By "substantial inhibition" and "substantially inhibit" is meant a decrease in enzyme activity below that acceptable for reliable, sensitive and reproducible assays of enzyme activity.

45 [0016] By "target nucleic acid sequence", "target nucleotide sequence" or "target sequence" is meant a specific nucleic acid sequence, or the nucleic acid sequence complementary thereto.

[0017] By "heterogeneous micelles" is meant hydrophobic aggregates comprising monomers of ionic and non-ionic detergent molecules in a liquid medium.

[0018] By "mixed micelles" is meant "heterogeneous micelles", as defined in this disclosure.

50 [0019] By "complementary" is meant having a nucleic acid sequence whereby stable hydrogen bonds are formed between the nucleotide bases of a region of one nucleic acid strand and those of a region of another nucleic acid strand under conditions suitable for discriminatory nucleic acid hybridization. That is, hydrogen bonds are most commonly formed between an adenosine(A) residue on one strand and a thymine (T) or uracil(U) residue on another strand, and between a guanine(G) residue on one strand and a cytosine (C) residue on another strand. Such regions of complementarity generally involve between about 12 and 100 or more contiguous nucleotides of each nucleic acid strand.

55 [0020] By "sufficiently complementary" is meant capable of forming a double-stranded hydrogen-bonded region with a target nucleic acid under hybridization conditions suitable to prevent a non-complementary nucleic acid from hybridizing thereto. While two nucleic acid strands are sufficiently complementary if they have 100% complementarity over



specific contiguous and corresponding regions, it is known to those skilled in the art that two single stranded nucleic acids having regions of less than 100% complementarity can form a double-stranded region under selective hybridization conditions. Such regions, not 100% complementary but able to form stable double stranded regions under these hybridization conditions, are hereby considered sufficiently complementary.

5 [0021] By "analogous" is meant a single-stranded nucleic acid region having a nucleotide sequence identical or similar to that of a second single-stranded nucleic acid to which it is being compared. This includes, for example, nucleic acids wherein the first nucleic acid contains a uracil residue in said region in place of a thymine present in the second nucleic acid and nucleic acid regions encoding or comprising functionally similar or identical biological agents or parts thereof.

10 [0022] By "sufficiently analogous" is meant having a nucleic acid sequence, as compared to a first single stranded nucleic acid, which allows a second single-stranded nucleic acid having that sequence to form a stable, hydrogen-bonded double-stranded region with a third nucleic acid under nucleic acid hybridization conditions, and wherein the third nucleic acid is sufficiently complementary to the first nucleic acid.

15 [0023] By "RNAse-inhibiting agent" is meant any agent capable of preventing the degradation of RNA by enzymes having RNAse activity. The term includes but is not limited to: enzymes such as proteases, cross-linking reagents, antibodies, and compounds which block the active site of the RNAse molecule.

[0024] By "nucleic acid" is meant polydeoxyribonucleotides or polyribonucleotides of at least two, and preferably 10 or more nucleotides in length. The term "nucleic acid" includes polynucleotides, oligonucleotides, and DNA or RNA molecules. The term "nucleic acid" can refer to either single-stranded or double-stranded polynucleotides, or both.

20 [0025] By "target nucleic acid" is meant a nucleic acid comprising a target nucleic acid sequence.

[0026] By "biological sample" or "test sample" is meant any specimen or sample containing substances derived at any time from living organisms containing nucleic acids. Such samples include, but are not limited to, food or agricultural samples; environmental samples; samples containing body fluids, secretions or excretions such as urine, blood, milk, cerebrospinal fluid, sputum, saliva, stool, lung aspirates, tears, lymphatic fluid, or semen; throat or genital swabs; and bacterial, viral, plant or animal cell cultures, suspensions or lysates.

25 [0027] By "amplification" or "target amplification" is meant increasing the number of target nucleic acid molecules having a target nucleic acid sequence.

[0028] Methods for detecting nucleic acids are well known in the art, and generally consist of contacting at least one labeled single-stranded nucleic acid probe with a single-stranded target nucleic acid under hybridization conditions, where the probe has a nucleic acid sequence sufficiently complementary to that of the target nucleic acid and the target nucleic acid has a specific sequence the presence of which is desired to be known; such methods are described in Maniatis, T., et al., Molecular Cloning: A Laboratory Manual (Cold Springs Harbor Laboratory 1982). Detection of double-stranded hybrid molecules depends on the nature of the label used; generally the probe incorporates a radioactive isotope such as  $^{32}\text{P}$ ,  $^3\text{H}$ , or  $^{14}\text{C}$ , or is conjugated with a fluorescent moiety, a hapten, or another non-radioactive reporter group. See e.g., Maniatis, supra, and Arnold et al., EP 0 309 230.

35 [0029] The sensitivity and reliability of diagnostic nucleic acid hybridization can be improved by using any one of a number of enzyme-mediated amplification systems to increase the copy number of the target nucleic acid sequence. Generally, such methods use the target nucleic acid as a template for at least one nucleic acid polymerase which may be used in concert with two or more nucleic acid primers in reiterative cycles to provide an exponential increase in the number of target nucleic acid sequences. Examples of amplification systems that are well known to those skilled in the art include the polymerase chain reaction (PCR) (Mullis et al., 155 Methods in Enzymology 335-50 (1987) and use of the double-stranded PCR products as templates for making multiple single-stranded RNA transcripts (Murakawa et al., 7 DNA 287-95 (1988)). Other amplification systems involve alternate rounds of DNA synthesis on an RNA template and transcription to amplify the target sequence. See e.g., Burg et al., WO 89/1050; Gingeras et al., WO 88/10315; Kacian and Fultz, EP 0 408 295. The preceding list is not intended to be an exhaustive listing of the various amplification methods, and other nucleic acid polymerases suitable for use in a nucleic acid amplification system are known to those skilled in the art. Following amplification, the resulting amplified nucleic acids can then be identified using a detection system as mentioned above.

40 [0030] Thus in a first aspect, the present method features a method for preventing an ionic detergent from denaturing or otherwise substantially inhibiting the activity of enzymes used in nucleic acid amplification reactions comprising: 1) target nucleic acids, 2) one or more nucleic acid primers, each one sufficiently complementary to a common target nucleic acid sequence to form double-stranded hydrogen-bonded regions with the target under suitably selective hybridization conditions, 3) necessary nucleoside triphosphates, salts, and other components necessary to achieve amplification, and 4) a quantity of an ionic detergent. The method of the present invention does not require a detergent removal step such as dialysis or chromatography. The method involves the addition of a non-ionic detergent to the sample and agitation of the sample in order to form mixed micelles of ionic and non-ionic detergent molecules. Following the formation of heterogeneous micelles, the nucleic acid polymerase and any other enzymes needed to conduct amplification can be added to the sample and the nucleic acid amplification reaction performed as usual.

[0031] In a second aspect of the present invention, a biological sample containing microorganisms or nucleic acids is contacted with a lysis or extraction buffer, and the released nucleic acids are stored in a buffer containing an ionic detergent such as LLS or SDS for subsequent amplification and use in a nucleic acid detection assay. Prior to the amplification step, a quantity of a non-ionic detergent is added to the extracted nucleic acid mixture either simultaneously with or preceding the addition of an effective quantity of ribo- or deoxyribonucleotide triphosphates, generally two or more nucleic acid primers, and any co-factors or metal ions necessary for the enzymatic reaction. The solution is then mixed vigorously, after which a nucleic acid polymerase is added to the mixture. The reaction mixture is incubated as appropriate for the amplification method used, for example a thermocycling or an isothermal amplification protocol. See e.g., American Society for Microbiology, Diagnostic Molecular Microbiology: Principles and Applications 56-70 (1993). The amplified target nucleic acids are then detected by hybridization methods as described above.

[0032] In another aspect, the method of the present invention may be used to prevent the inhibition of other enzymes commonly used in industry, medical clinics, research laboratories and commercial formulations. For example, the method of the present invention may be used to prevent the inhibition of a restriction enzyme digestion of a nucleic acid in a sample containing an ionic detergent without the need to remove the ionic detergent from solution first.

[0033] While the foregoing disclosure describes the problem solved by the present invention and its general means of solution, it will be understood that the foregoing disclosure does not in any way limit the present invention or its application, which are defined solely by the claims which follow this disclosure.

#### SUMMARY OF THE INVENTION

[0034] The present invention features a method for conducting a enzymatic reaction in a solution containing an ionic detergent, such as sodium dodecyl sulphate (SDS) or lithium lauryl sulphate (LLS), at a concentration which is normally sufficient to inhibit the activity of the enzyme. In one embodiment, the present invention relates to a method for preventing the inhibition of at least one enzyme in a nucleic acid target amplification reaction. The method concerns the addition to a solution containing an ionic detergent (preferably SDS or LLS) and nucleic acids to be analyzed; of, generally, two or more different oligonucleotide primers; one primer having a sequence sufficiently complementary and the other a sequence sufficiently analogous to a target nucleic acid sequence; four different nucleotides; necessary salts and cofactors such as  $MgCl_2$  or NaCl; and an amount, between 6-20% (v/v), of a non-ionic detergent, preferably polyoxyethylene (20) sorbitan monoalkylates (the Tween® series of detergents) or polyoxyethylene p-t-octyl phenol derivatives (e.g., Triton® X-100 and Triton® X-102). These constituents are mixed, and a nucleic acid polymerase, preferably a heat-stable DNA polymerase such as the DNA polymerase from the bacterium *Thermus aquaticus* (Taq DNA polymerase), is added to the reaction mixture and the mixture is subjected to a nucleic acid amplification procedure, preferably the polymerase chain reaction (PCR). In another preferred embodiment two enzymes are used in the amplification reaction: a reverse transcriptase having RNase H activity, such as MMLV RT, and a RNA polymerase, such as T7 polymerase.

[0035] Another embodiment uses a DNA polymerase derived from *Bacillus stearothermophilus*.

[0036] In another preferred embodiment, the method of the present invention is used to prevent inhibition of a restriction enzyme in the presence of sodium dodecyl sulfate (SDS). Plasmid pUC19 (Bethesda Research Laboratories, Gaithersburg, MD) was digested with restriction endonuclease Eco R1 at the single Eco R1 site. Following linearization, the plasmid DNA was radiolabeled with  $[\alpha\text{-}^{32}\text{P}]$  dATP using the Klenow fragment of *E. coli* DNA polymerase I. The radiolabeled probe was precipitated and resuspended in a solution containing differing amounts of SDS and Tween®-20. The DNA was then given an excess of restriction endonuclease Pvu II, and incubated for one hour at 37°C. The resulting samples were analyzed on a 7% polyacrylamide gel under non-denaturing conditions.

[0037] Although hydrophobic relationships probably play an important role in the deinhibition of the enzymatic reactions by removing enzyme-inhibiting ionic detergents from solution, micelle formation alone may not be the only determinant as to the suitability of a particular non-ionic detergent in the method described herein. Thus, while the formation of mixed micelles is thought by the Applicants to be important in the practice of this invention, the Applicants remain uncertain as to the precise mechanism which allows enzyme activity to be preserved in the presence of ionic detergent.

[0038] It is an object of the present invention to provide a method for conducting a target amplification reaction in the presence of normally inhibiting or denaturing amounts of an ionic detergent such as LLS or SDS.

[0039] It is also an object of the present invention to provide a method for conducting a qualitative and/or a quantitative analysis of a biological or clinical sample containing nucleic acids and an ionic detergent such as LLS or SDS, wherein such an analysis involves at least one enzyme-mediated reaction, such as the amplification of a specific nucleic acid sequence or a restriction digest, without the need to separate the nucleic acid analyte from the ionic detergent before commencing the reaction.

[0040] It is another object of the present invention to provide a method for preventing the inhibition of a nucleic acid amplification reaction in a test sample wherein the sample includes: 1) nucleic acids to be tested for the presence or absence of a specific nucleic acid sequence; and 2) from about 0.1-1.5% of an ionic detergent, preferably about

0.3-0.7% SDS or LLS, and where the enzyme or enzymes to be employed in the nucleic acid amplification reaction are preferably Taq DNA polymerase, T7 DNA polymerase, and/or retroviral RNA-directed DNA polymerase (reverse transcriptase or RT); most preferably Taq DNA polymerase. The method comprises the steps of adding non-ionic detergent, between about 6-20% of polyoxyethylene (20) sorbitan monolaurate (Tween® -20) or polyoxyethylene (9) p-t-octyl phenol (Triton® X-100) to the nucleic acids and ionic detergent, mixing the solution together vigorously to form heterogeneous detergent micelles, adding an effective amount of a nucleic acid polymerase, preferably between about 2-8 units of Taq DNA polymerase and subjecting the reaction mixture to a nucleic acid amplification procedure, such as PCR.

[0041] It is further an object of this invention to provide a kit for either the qualitative analysis or quantitative analysis, or both, of biological samples containing nucleic acids or microorganisms which contain nucleic acids. When the sample contains microorganisms, such a kit should provide a lysis, permeabilization, or sample transport reagent including between about 0.1%-1.5% of an ionic detergent, preferably SDS or LLS, to inhibit protease and nuclease activity and to assist in dissolution of the cell wall and membrane. In such a reagent, the liberated nucleic acids are protected from nuclease degradation by the ionic detergent. The kit should also provide a second reagent containing: 1) generally two or more nucleic acid primers having nucleic acid sequences sufficiently complementary to that of a target nucleic acid sequence to form, under hybridization conditions, a hydrogen-bonded region with either or both of the complementary nucleic acid strands having such a target sequence; 2) from about 0.5-10 mM  $MgCl_2$  or  $MnCl_2$ ; 3) an effective amount of a disulfide cleaving agent, preferably from about 0.5-10 mM dithiothreitol (DTT); and 4) from 6 to 20% (v/v) of a non-ionic detergent, preferably polyoxyethylene (20) sorbitan monolaurate (Tween® -20) or polyoxyethylene (20) sorbitan monooleate (Tween® -80). An aliquot of the sample solution containing the liberated nucleic acids in the lysis, permeabilization or transport medium is added to the second solution, and mixed vigorously. From about 2-8 units of at least one nucleic acid polymerase, preferably Taq DNA polymerase, is then added to the reaction mixture and the mixture is treated according to the normal procedure for the polymerase chain reaction. Following this, the sample is tested for the presence or absence of at least one specific nucleic acid sequence using nucleic acid hybridization methods and a labeled nucleic acid probe.

[0042] It is yet another object of the present invention to provide a method for the amplification of nucleic acids in the presence of an ionic detergent, preferably about 0.3 to 0.7% LLS. In this method, the final composition of the starting solution includes the following: 1) about 50 mM Tris-HCl (pH 7.6), 17.5 mM  $MgCl_2$ , 25 mM KCl and 2 mM spermidine, 2) about 25 mM each of four different ribonucleotide triphosphates (A, U, G and C) and four different deoxyribonucleotide triphosphates (A, T, G and C), 3) about 1 mM DTT, 4) about 20 picomoles of, generally, two or more single-stranded nucleic acid primers which have nucleic acid sequences sufficiently complementary to that of a target nucleic acid sequence to form a double-stranded, hydrogen-bonded region with either a positive or a negative sense nucleic acid strand having such a target nucleic acid sequence, or both, under hybridization conditions, 5) between about  $6 \times 10^{-3}$  to  $6 \times 10^{-9}$  picomoles of a nucleic acid having at least one copy of a specific target nucleic acid sequence.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0043] The claimed method and kit feature a series of steps for conducting a target amplification reaction in the presence of an ionic detergent, as well as a combination of reagents for accomplishing such steps.

[0044] In one of its preferred embodiments the present invention provides a method and means for conducting a nucleic acid amplification reaction when the sample to be analyzed contains ionic detergents. The method involves adding a quantity of a non-ionic detergent to the sample solution and mixing the ionic and the non-ionic detergents together vigorously before the addition of the enzyme or enzymes and initiation of the amplification reaction.

[0045] While the Applicants used purified nucleic acids in some of their embodiments, any source of nucleic acids, either purified or unpurified can be used so long as it contains or is suspected of containing the target nucleic acid sequence. This sequence may be present in DNA or RNA, which may be single-stranded or double-stranded. A mixture of these nucleic acids may be used, as may the nucleic acids from a previous amplification reaction. This invention is therefore a generally useful method for overcoming the inhibitory effects of a detergent in a sample containing nucleic acids to be analyzed.

#### Materials

[0046] The non-ionic detergents Tween® -20, Tween® -40, Tween® -80, Triton® X-100 and Triton® X-102 were purchased from the Sigma Chemical Co., St. Louis, Mo.

[0047] The nucleic acid primers were synthesized by use of standard phosphoramidite chemistry; various such methods are well known in the art, see e.g., Caruthers et al., 154 Methods in Enzymology 287 (1987); Bhatt, WO 90/11322, and Klem et al., PCT WO92/07864. Applicants prepare the probes using a Model 380A DNA synthesizer (Applied Biosystems, Inc., Foster City, CA).

Detection Method

[0048] The amplification products were quantified using a detectable chemiluminescent acridinium ester-labeled nucleic acid probe that hybridizes to the target sequence. In particular, a double-stranded hybrid molecule is detected by contacting the amplified sample with the labeled probe under specified hybridization conditions, selectively hydrolyzing the acridinium ester bound to unhybridized probe, and measuring the chemiluminescence of the remaining acridinium ester (i.e. that associated with double-stranded nucleic acid regions) in a luminometer. See e.g., Arnold et al., EP 0 309 230, and Nelson et al., in *Nonisotopic DNA Probe Techniques* 275 (Academic Press, San Diego 1992).

[0049] The following examples do not necessarily represent optimal conditions for the use of the present invention; they are intended to be exemplary only and represent currently preferred embodiments of the present invention. These examples are not intended to be limiting as to the scope of possible embodiments of the claimed method, such embodiments being immediately apparent to those of skill in the art upon exposure to the present disclosure.

Example 1

[0050] This example demonstrates the effectiveness of using the present method in a transcription-based amplification system, see Kacian & Fultz, *supra*. The target nucleic acid for this example was a solution of total ribosomal RNA purified from *Ureaplasma urealyticum* (0.6 picomoles/ml) in a buffer containing 30 mM sodium phosphate pH (6.7), 1.0 mM disodium EDTA (ethylenediaminetetracetic acid disodium), 1 mM EGTA (ethylene glycol-bis ( $\beta$ -aminoethyl ether) N,N,N',N'-tetracetic acid), and 110 mM (3.0% w/v) LLS. Serial dilutions were made in the same buffer.

[0051] A solution of 25% (v/v) Triton® X-102 in deionized water was prepared. Reaction volumes were 100  $\mu$ l, and reaction was conducted in microcentrifuge tubes. Each tube was given 10  $\mu$ l of a buffer containing 500 mM Tris-HCl (pH 7.6), 175 mM MgCl<sub>2</sub>, 250 mM KCL, and 20 mM spermidine, 0.5  $\mu$ l of 1 M DTT, 10  $\mu$ l of a solution of ribonucleotides containing 25 mM rCTP and rUTP and 65 mM rATP and rGTP, 2  $\mu$ l of a solution containing 100 mM each of dATP, dTTP, dCTP, and dGTP, 8.6  $\mu$ l of a solution of 30 pM of the first primer (81(-); SEQ ID NO: 1), 3.4  $\mu$ l of a solution of 30 pM of the second primer (uur C 1(+); SEQ ID NO: 2), 10  $\mu$ l of undiluted, 1:10 diluted, or 1:100 diluted target rRNA prepared as described above, varying amounts of the 25% (v/v) Triton® X-102 solution, and water to a volume of 93.6  $\mu$ l. The sequences of these primers are as follows:

SEQ ID NO 1:

5  
AATTTAATACGACTCACTATAGGGAGAGCGTAGCGATGACCTATTTTACTTGC-3'

SEQ ID NO 2:

5'-TGTAGTGATCATATCAGAGTGG-3'

[0052] Each reaction tube was mixed vigorously using a vortex mixer, and then the samples were heated to 95°C for 2 minutes and cooled to room temperature. Moloney Murine Leukemia Virus RNA-directed DNA polymerase (MMLV reverse transcriptase, 300 units) and 400 units of T7 RNA polymerase were separately added to each tube in a total volume of 6.44  $\mu$ l. The samples were then incubated at 37°C for 4 hours. Tubes were cooled to 4°C awaiting the detection step.

[0053] Quantification of amplified target sequences was performed according to the HPA chemiluminescence method of Arnold et al., *supra*. One of two labeled probes specific to the target sequence were used in the hybridization assay. Their sequences are as follows:

SEQ ID NO:3

5'-GTGATCATATCAGAGTGGAAATACCTGTTCCCATCC-3'

SEQ ID NO: 4

5' -GCTTGTGTCTTCAGTTCGTGAGATCTCGGC-3'

## Example 2

[0054] This example demonstrates the ability of a polyoxyethylene(9) p-t-octyl phenol derivative (Triton® X-102) to act as the non-polar detergent in the method of the present invention. The concentration of LLS was 0.3% (w/v). Triton® X-102 concentrations were 9%, 10%, and 11% (v/v). Duplicate experiments were run for each condition, and duplicate HPA detections made for each experiment; the resulting values were averaged. Amount of amplification is proportional to the amount of label detected; this quantity is given in RLU or relative light units. Values appearing in parentheses were control experiments. The results of the amplification experiments are shown below in Table 1.

TABLE 1

Amplification Reaction in Presence of 0.3% (w/v) LLS and Triton® X-102 [Average Relative Light Units (RLU)]			
	Input Target RNA (picomoles)		
	6 X 10 <sup>-3</sup>	6 X 10 <sup>-4</sup>	6 X 10 <sup>-5</sup>
No LLS, no Triton® X-102	(5.2 X 10 <sup>7</sup> )		
No Target RNA	(100)		
10% Triton® X-102; no LLS	(1.7 X 10 <sup>8</sup> )		
9% Triton® X-102	24,000	770	530
10% Triton® X-102	72,000	280	100
11% Triton® X-102	78,000	87,000	87,000

## Example 3

[0055] The following experiment demonstrates the sensitivity of nucleic acid amplification according to the presently claimed method. The experiment was conducted as in Example 1, with the following exceptions. Tween® -20 was substituted for Triton® X-102, and the amounts of added target RNA were 6 X 10<sup>-3</sup>, 6 X 10<sup>-5</sup>, 6 X 10<sup>-7</sup> and 6 X 10<sup>-9</sup> picomoles. Final concentrations of Tween® -20 were 13, 15 and 20% (v/v). The results are shown in Table 2.

TABLE 2

Sensitivity of Amplification Reaction in Presence of 0.3% (w/v) LLS and 20% (v/v) Tween® -20 [Average Relative Light Units (RLU)]				
	Input Target RNA (picomoles)			
	6 X 10 <sup>-3</sup>	6 X 10 <sup>-4</sup>	6 X 10 <sup>-5</sup>	6 X 10 <sup>-9</sup>
No LLS, no Tween® -20		2.3 X 10 <sup>6</sup>	2.3X 10 <sup>6</sup>	2.2 X 10 <sup>6</sup>
LLS, Tween® ; no target	(6,300)			
13% Tween® -20	2.4 X 10 <sup>6</sup>	1.9 X 10 <sup>6</sup>	1.3 X 10 <sup>6</sup>	1.2 X 10 <sup>6</sup>
15% Tween® -20	2.3 X 10 <sup>6</sup>	2.2 X 10 <sup>6</sup>	1.2 X 10 <sup>6</sup>	940,000
20% Tween® -20	2.3 X 10 <sup>6</sup>	1.6 X 10 <sup>6</sup>	310,000	320,000

## Example 4

[0056] This example demonstrates the effectiveness of the present method for use with nucleic acid amplification employing the polymerase chain reaction (PCR). For this experiment the target nucleic acid was a restriction fragment having a known DNA sequence obtained from a plasmid containing the E6 gene of Human Papilloma Virus. The two oligonucleotide primers (SEQ ID NO:4 AND SEQ ID NO: 5) were designed to be complementary to opposite strands of the double-stranded target nucleic acid. The sequences of these primers are as follows:  
SEQ ID NO: 5

5' -GCAATGTAGGTGTATCTCC-3'

5 SEQ ID NO: 6

5' -TATGCACAGAGCTGCA-3'

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[0057] The final reaction volume for each sample was 100  $\mu$ l. In this experiment, 2 drops of mineral oil were added to each tube, followed by a precalculated amount of water. 1  $\mu$ l of target DNA in 100 mM Tris-HCl pH 7.6, 10 mM EDTA (1 X 10<sup>4</sup> copies of the target sequence) was then added. A buffer containing 30 mM sodium phosphate pH 6.7, 1.0 mM disodium EDTA, 1 mM EGTA, and 110 mM (3.0% w/v) LLS was added to some tubes to give a final LLS concentration of 0.3%. Differing volumes of a 50% (v/v) solution of Tween® -20 was also added to some tubes to give a reaction concentration of 10%, 12%, 14%, 16%, 18%, 20%, 22%, or 24%. Following the addition of detergent the samples were mixed on a vortex mixer.

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[0058] A PCR premix was made separately, containing 10  $\mu$ l of 10X PCR buffer, 20 nanomoles of each deoxyribonucleotide, 100 picomoles of each nucleic acid primer and 2.5 units of Taq DNA polymerase for each reaction tube. The sample tubes were placed on ice, and the PCR premix was added to each sample and mixed. Each sample was heated to 94°C for 3 minutes, then taken through 35 thermocycles comprising the following: incubation at 51°C for 30 seconds, followed by incubation at 72°C for 2 minutes, and finally at 94°C for 1 minute. Following the final 94°C incubation, the samples were incubated at 72°C for 5 minutes then stored at 4°C overnight for detection.

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[0059] Detection was carried out as in Example 1 using a probe complementary to the target nucleic acid sequence.

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The sequence of the probe is as follows:

SEQ ID NO: 7

5' -GAACAGCAATACAACAACCGTTGTGTG-3'

30

[0060] The results are shown in Table 3.

TABLE 3

35

PCR Amplification in the Presence of 0.3% (w/v) LLS	
	Average RLU
No LLS, no Tween®	290,000
10% Tween® -20	310,000
12% Tween® -20	250,000
14% Tween® -20	360,000
16% Tween® -20	160,000
18% Tween® -20	320,000
20% Tween® -20	180,000
22% Tween® -20	120,000
24% Tween® -20	140,000

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45

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Example 5

[0061] This experiment shows the effectiveness of Tween® -20 to prevent the inhibition of Taq DNA polymerase-mediated PCR at different concentrations of LLS and Tween® -20. The experiment was conducted as in Example 3, except as follows: LLS concentrations were made 0%, 0.3%, 0.5%, and 0.7% (w/v). Tween® -20 concentrations were variously 0%, 10%, 14%, 18%, and 20% (v/v). The results are shown in Table 4.

55

TABLE 4

PCR Amplification in Presence of Various Concentrations of LLS and Tween® -20 [Average RLU]				
	0% LLS	0.3% LLS	0.5% LLS	0.7% LLS
0% Tween® -20	410,000	14	25	45
10% Tween® -20	120,000	11,000	290	300
14% Tween® -20	170,000	170,000	64,000	280
18% Tween® -20	230,000	210,000	77,000	340
20% Tween® -20	170,000	190,000	42,000	310

Example 6

[0062] This experiment was performed as is Example 4, except Tween® -40 (polyoxyethylene (20) sorbitan monopalmitate) was substituted for Tween® -20. The results are shown in Table 5.

TABLE 5

PCR Amplification in Presence of Various Concentrations of LLS and Tween® -40 [Average RLU]				
	0% LLS	0.3% LLS	0.5% LLS	0.7% LLS
0% Tween® -40	2,500,000	48,000	4,900	5,100
10% Tween® -40	160,000	260,000	5,400	5,100
14% Tween® -40	180,000	110,000	65,000	5,200
18% Tween® -40	150,000	82,000	52,000	17,000
20% Tween® -40	77,000	49,000	32,000	6,800

Example 7

[0063] This experiment was conducted as in Example 4, except Tween® -80 (polyoxyethylene (20) sorbitan monooleate) was substituted for Tween® -20. Experimental compositions which were not tested are indicated "ND". The results are shown in Table 6.

TABLE 6

PCR Amplification in Presence of Various Concentrations of LLS and Tween® -80 [Average RLU]				
	0% LLS	0.3% LLS	0.5% LLS	0.7% LLS
0% Tween® -80	2,500,000	ND	0.0	ND
10% Tween® -80	2,200,000	61,000	1,100,000	230,000
14% Tween® -80	690,000	260,000	130,000	0.0
18% Tween® -80	900,000	750,000	140,000	94
20% Tween® -80	330,000	460,000	39,000	260,000

Example 8

[0064] This experiment demonstrates the effectiveness of the present method for use with biological samples suspected of containing nucleic acids or nucleic acid-containing microorganisms. The sample was a pool of endo-cervical swab specimens in a buffer containing 30 mM sodium phosphate (pH 6.8), 1.0 mM disodium EDTA (ethylenediamine-tetracetic acid disodium), 1 mM EGTA (ethylene glycol-bis (β-aminoethyl ether) N,N,N',N'-tetracetic acid), and 110 mM (3.0% w/v) LLS which tested positive for Chlamydia trachomatis in a previous direct nucleic acid hybridization assay without amplification.

[0065] The samples were treated with 40 μl of a Proteinase K solution (0.1 unit/μl) for each 800 μl of sample, and

incubated at 60°C for 20 minutes. Tween® -80 (polyoxyethylene (20) sorbitan monooleate) was added to a sample dilution buffer containing 40 mM Tris-HCl (pH 8.0), 10 mM N-acetyl-L-cysteine (NALC), and 2 mM EDTA. Amounts of Tween® -80 were added to this buffer in order to make the final Tween® concentration during the amplification 6.4%, 6.8%, 7.2%, 7.6%, 8%, 10% and 12%. Ten microliters of the specimen sample was combined with 40 µl of each concentration of non-ionic detergent in dilution buffer and mixed vigorously using a vortex mixer, then stored for either 3-4 days at 4°C.

[0066] 25 µl of an amplification buffer containing 20% (w/v) polyvinylpyrrolidone, 16 mM each of rCTP, rATP and rUTP and 20 mM rGTP, 6 mM deoxyribonucleotides, 160 mM Tris pH 7.5, 92 mM MgCl<sub>2</sub>, 92 mM KCl, 3.0 picomoles of a first primer (T7 AproCtB(-)1519; SEQ ID NO: 7) and 25.0 picomoles of a second primer (CtB(+)-1428b; SEQ ID NO: 8) was pipetted into a separate tube for each experiment, and 200 µl of mineral oil was layered on top of each tube. One of the oligonucleotide primers had a nucleic acid sequence analogous to for a region of Chlamydia trachomatis ribosomal RNA; the other oligonucleotide primer had a nucleic acid sequence complementary to a region of Chlamydia trachomatis ribosomal RNA. The sequences of these primers are as follows:  
SEQ ID NO: 8:

5' -AATTTAATACGACTCACTATAGGGAGACCCGAAGATTCCCCTTGATCGC-3'

SEQ ID NO: 9:

5' -CGGAGTAAGTTAAGCACGCGGACGATTGGAAGA-3'

[0067] The 50 µl containing each sample and detergent combination was pipetted through the layered mineral oil into the amplification buffer. Each tube was heated at 95°C for 5 minutes in a heating block, then transferred to a 42°C heating block for another 5 minutes before the addition of enzyme. 25 µl of an enzyme solution containing 900 units Moloney Murine Leukemia Virus reverse transcriptase (MMLV RT) and 400 units T7 RNA polymerase was added to each tube and mixed into solution. Each tube was then incubated for 42°C for 1 hour, then treated with 20 µl of a solution containing 50 units of essentially RNase-free DNase and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) in ethanol. Samples were incubated for another 10 minutes at 42°C and HPA detection was carried out as described above using a labeled oligonucleotide probe specific for the target Chlamydia trachomatis ribosomal RNA. The sequence of the probe is as follows:

SEQ ID NO: 10

5' -AGAGTCCGTAGAGCGATGAGAACG-3'

[0068] Positive controls are indicated in parentheses, and were samples in which the indicated amount of RNA, not contained in a biological sample, and in a final concentration of 0.3% LLS, was subjected to the amplification procedure. Each experimental sample was run in duplicate. The results are indicated in the Table 7.

TABLE 7

Amplification of Nucleic Acids in Biological Samples Stored in Presence of Heterogeneous Micelles [Average RLU]	
	<u>C. trachomatis</u> positive pool in 0.3% LLS
No target	1600
0.5 fg <u>C. trachomatis</u> rRNA	2,000,000
5.0 fg <u>C. trachomatis</u> rRNA	1,300,000
Sample in 6.4% Tween® -80	150,000
Sample in 6.8% Tween® -80	2,400,000
Sample in 7.2% Tween® -80	2,300,000
Sample in 7.6% Tween® -80	2,800,000
Sample in 8.0% Tween® -80	2,900,000



TABLE 7 (continued)

Amplification of Nucleic Acids in Biological Samples Stored in Presence of Heterogeneous Micelles [Average RLU]	
	<i>C. trachomatis</i> positive pool in 0.3% LLS
Sample in 10% Tween® -80	3,000,000
Sample in 12% Tween® -80	2,900,000

**Example 9**

[0069] This example illustrates the effectiveness of the method of the present invention to prevent the inhibition of enzyme-mediated reactions by ionic detergents in systems other than nucleic acid amplification reactions. 5 µg of plasmid pUC19 (Bethesda Research Laboratories, Gaithersburg, MD) were digested with restriction endonuclease Eco R1 at its sole Eco R1 site in a reaction mixture containing 5 µl of 10X High Salt buffer (10 mM NaCl, 50 mM Tris (pH 7.5), 10 mM MgCl<sub>2</sub> and 1 mM DTT), 48 units of Eco R1 (48 units/µl) in a final reaction volume of 50 µl. The reaction mixture was incubated at 37°C for 1 hour. The sample was then precipitated with a solution containing 300 µl of ethanol, 5 µl of 3 M sodium acetate, and 1 µl of glycogen and redissolved in 16 µl water.

[0070] Following linearization of the circular plasmid, the plasmid was radiolabeled with [α]-<sup>32</sup>P dATP using the Klenow fragment of *E. coli* DNA polymerase I as follows. The dissolved DNA was given 2 µl of High Salt buffer, 1 µl of [α]-<sup>32</sup>P dATP, and 1 µl of the Klenow fragment (5 units/µl) (Stratagene, San Diego, CA). The reaction mixture was incubated for 20 minutes at room temperature, then precipitated by adding 2 µl of 3 M sodium acetate and 200 µl ethanol, and pelleted in a microcentrifuge. The DNA was redissolved in 100 µl of water.

[0071] To test whether the addition of Tween® -20 to a solution containing SDS and the radiolabeled DNA would prevent inhibition of digestion of the linearized DNA by restriction endonuclease Pvu II, different reaction mixtures were made up with varying amounts of the two detergents, as shown below. Each reaction was in a total volume of 50 µl. SDS and Tween® -20 were added to the reaction mixture prior to the addition of the radiolabeled DNA. 5 µl of 10X REACT buffer (500 mM Tris-HCl (pH 7.4), 60 mM MgCl<sub>2</sub>, 500 mM NaCl, and 500 mM KCl) were also added to the reaction mixture. Ten units of Pvu II (10 units/µl) were then added to each tube, along with 1 µl (50 ng) of the radiolabeled DNA. The reaction mixture was incubated at 37°C for 1 hour. 5 µl of each digestion mixture was added to 10 µl of non-denaturing gel loading buffer and 5 µl of water, and an aliquot of each sample was analyzed on a 7% polyacrylamide non-denaturing gel. Molecular weight markers were radiolabeled φX174 DNA digested with Hinf I. The gel was run until the running dye had reached the bottom of the gel, and the gel was used to expose X-ray film for either 4 hours or overnight. The X-ray film was exposed (Figure 1), and the results were analyzed.

PERCENT SDS IN EACH SAMPLE		
Gel Lanes	Percent SDS	µl 2% SDS Solution
1-5	0	0
6-10	0.01	2.5 (10X dilution)
11-15	0.05	1.25
16-20	0.1	2.5
21-25	0.5	12.5

PERCENT TWEEN® -20 IN EACH SAMPLE		
Gel Lane	Percent Tween® -20	µl 50% (v/v) Tween® -20
1,6,11,16,21	0	0
2,7,12,17,22	10	10
3,8,13,18,23	14	14
4,9,14,19,24	18	18
5,10,15,20,25	20	20

[0072] The gel lanes are numbered from left to right. The two leftmost lanes are the molecular weight markers, with their sizes (in number of bases) indicated next to the bands in the overnight exposure, followed by a negative control of Pvu II undigested, Eco R1 linearized pUC19 DNA. The lane to the far right also contains the molecular weight markers.

[0073] The Eco R1 site in the 2686 bp pUC19 plasmid is at map position 396. The Pvu II sites in pUC19 are at map positions 306 and 628. Thus, a complete digestion of the pUC19 DNA would yield expected fragments of 90, 232, and 2364 bp in length.

[0074] The results, shown in Figure 1, indicate that Pvu II digestion is inhibited by concentrations of SDS as low as 0.01% (w/v). However, the addition of 10-20% Tween® -20 to the reaction mixture before addition of the enzyme allows the restriction digestion to occur.

[0075] The foregoing examples demonstrate currently preferred embodiments of the present invention. However, it will be immediately appreciated by those skilled in the art in light of this disclosure that other combinations of enzymes, ionic detergents and non-ionic detergents may be used in the method of the present invention. Other such combinations may be screened for use in the present invention without undue experimentation using the methods disclosed herein in conjunction with standard enzyme assay procedures.

# SEQUENCE LISTING

[0076]

## (1) GENERAL INFORMATION:

### (i) APPLICANT:

- (A) NAME: GEN-PROBE INCORPORATED
- (B) STREET: 9880 Campus Point Drive
- (C) CITY: San Diego
- (D) STATE: California
- (E) COUNTRY: USA
- (F) POSTAL CODE (ZIP): 92121

(ii) TITLE OF INVENTION: Methods for Suppressing Inhibition of Enzyme-Mediated Reactions by Ionic Detergents

(iii) NUMBER OF SEQUENCES: 10

### (iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

### (v) CURRENT APPLICATION DATA:

APPLICATION NUMBER: EP 95103520.3

## (2) INFORMATION FOR SEQ ID NO: 1:

### (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 53 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

### (ii) MOLECULE TYPE: DNA (genomic)

### (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

AATTTAATAC GACTCACTAT AGGGAGAGCG TAGCGATGAC CTATTTTACT 50  
TGC 53

5 (2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

20 TGTAGTGATC ATATCAGAGT GG 22

25 (2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 36 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GTGATCATAT CAGAGTGGAA ATACCTGTTC CCATCC 36

40 (2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

55 GCTTGTGTCT TCAGTTCGTG AGATCTCGGC 30

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GCAATGTAGG TGTATCTCC

19

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

TATGCACAGA GCTGCA

16

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GAACAGCAAT ACAACAAACC GTTGTGTG

28

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 49 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

5 AATTTAATAC GACTCACTAT AGGGAGACCC GAAGATTCCC CTTGATCGC 49

(2) INFORMATION FOR SEQ ID NO: 9:

10

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33 base pairs

(B) TYPE: nucleic acid

15

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CGGAGTAAGT TAAGCACGCG GACGATTGGA AGA 33

25

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

30

(A) LENGTH: 24 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

35

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

40

AGAGTCCGTA GAGCGATGAG AACG 24

#### 45 Claims

1. A method for enhancing enzymatic activity in a liquid containing an ionic detergent and at least one enzyme substrate, comprising the steps of:

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a. providing a liquid containing at least one ionic detergent present in an amount sufficient to inhibit an enzymatic activity and at least one enzyme substrate;

b. mixing a non-ionic detergent into the liquid to a final concentration between about 6-20% (v/v) of said non-ionic detergent thereby producing a mixture and suppressing an inhibitory effect of the ionic detergent on the enzymatic activity;

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c. adding to the mixture at least one enzyme having the enzymatic activity; and

d. incubating the mixture under reaction conditions favorable to the enzymatic activity.

2. The method of claim 1, wherein the liquid comprises a biological sample.

3. The method of claim 1 or 2, wherein the enzyme substrate comprises a nucleic acid.
4. The method of any one of claims 1 to 3, wherein the enzyme is selected from the group consisting of a DNA-directed DNA polymerase, an RNA-directed DNA polymerase, a DNA-directed RNA polymerase, an RNA-hydro-  
5 lyzing enzyme, a restriction endonuclease and a protease.
5. The method of claim 4, wherein the enzyme is selected from the group consisting of a retrovirus-derived reverse transcriptase, a DNA polymerase derived from *Thermus aquaticus* or from *Bacillus stearothermophilus*, a restriction  
10 endonuclease and T7 RNA polymerase.
6. The method of any one of claims 1 to 5, wherein the ionic detergent comprises a composition selected from the group consisting of water-soluble lauryl sulfate salts.
7. The method of claim 6, wherein the ionic detergent is lithium lauryl sulfate or sodium dodecyl sulfate.
8. The method of any one of claims 1 to 7, wherein the ionic detergent is at a final concentration of between about  
15 0.1% and 0.7% (w/v).
9. The method of any one of claims 1 to 8, wherein the non-ionic detergent is a polyoxyethylene (20) sorbitan mono-  
20 alkylate derivative or a polyoxyethylene p-t-octyl phenol derivative.
10. The method of claim 9, wherein the non-ionic detergent is polyoxyethylene (20) sorbitan monolaurate, polyoxyeth-  
ylene (20) sorbitan monopalmitate, polyoxyethylene (20) sorbitan monostearate, polyoxyethylene (20) sorbitan  
25 monooleate or polyoxyethylene (9) p-t-octyl phenol derivative.
11. A method for detecting a nucleic acid in a biological sample, comprising the steps of:
  - a) contacting a biological sample containing a target nucleic acid sequence with an ionic detergent;
  - b) then mixing a non-ionic detergent at a final concentration of between about 6-20% (v/v) with the biological  
30 sample, thereby suppressing an inhibitory effect of the ionic detergent on enzymatic activity;
  - c) adding at least one nucleic acid primer having a nucleic acid sequence capable of hybridizing to the target  
nucleic acid sequence, nucleotide triphosphates, necessary cofactors or salts, and at least one enzyme having  
nucleic acid polymerase activity, thereby producing a mixture;
  - d) incubating the mixture under conditions sufficient to cause amplification of the target nucleic acid sequence,  
35 thereby producing an amplified target nucleic acid sequence; and
  - e) detecting the amplified target nucleic acid sequence.
12. The method of claim 11, wherein the ionic detergent comprises a composition selected from the group consisting  
40 of water-soluble lauryl sulfate salts.
13. The method of claim 12, wherein the ionic detergent is lithium lauryl sulfate or sodium dodecyl sulfate.
14. The method of any one of claims 11 to 13, wherein the ionic detergent is at a final concentration of between about  
45 0.1-0.7% (w/v).
15. The method of any one of claims 11 to 14, wherein the non-ionic detergent comprises a composition selected from  
the group consisting of polyoxyethylene (20) sorbitan mono-alkylate derivatives and polyoxyethylene p-t-octyl phe-  
nol derivatives.
16. The method of claim 15, wherein the non-ionic detergent is polyoxyethylene (20) sorbitan monolaurate, polyox-  
50 yethylene (20) sorbitan monooleate, polyoxyethylene (20) sorbitan monopalmitate, polyoxyethylene (20) sorbitan  
monostearate or a polyoxyethylene (9) p-t-octyl phenol derivative.
17. The method of any one of claims 11 to 16, wherein the target nucleic acid sequence comprises at least one se-  
55 quence specific to ribosomal RNA or ribosomal DNA.
18. The method of claim 17, wherein the ribosomal RNA sequence is specific for *Chlamydia trachomatis*, *Ureaplasma  
urealyticum* or human cells infected with Human Papilloma Virus.

19. The method of claim 11, wherein the mixing step adds the non-ionic detergent simultaneously with or preceding addition of metal ions necessary for enzymatic reaction.
- 5 20. The method of any one of claims 11 to 19, wherein the adding step further comprises adding an RNase-inhibiting agent, and, optionally, inactivating the RNase-inhibiting agent before adding at least one enzyme having nucleic acid polymerase activity.
21. The method of claim 20, wherein the RNase-inhibiting agent is a protease.
- 10 22. The method of claim 21, wherein the protease is proteinase K.
23. The method of any one of claims 20 to 22, wherein the step of inactivating the RNase-inhibiting agent comprises heating the mixture to between about 90°C to 100°C for between about 1 to 5 minutes.
- 15 24. A kit for amplifying a target nucleic acid in a biological sample, said kit comprising:
  - a) a first reagent for collecting and transporting a biological sample containing a target nucleic acid sequence, comprising an ionic detergent at a final concentration of between about 0.1%-1.5% (w/v) ;
  - 20 b) a second reagent containing a non-ionic detergent for mixing with the first reagent such that the non-ionic detergent is at a final concentration between about 6-20% (v/v) ;
  - c) an amplification reagent comprising at least one primer capable of hybridizing to the target nucleic acid sequence, nucleotide triphosphates, and cofactors or salts required for nucleic acid amplification; and
  - 25 d) at least one enzyme having nucleic acid polymerase activity for initiating nucleic acid amplification of the target nucleic acid sequence.
- 25 25. The kit of claim 24, wherein the ionic detergent is a lauryl sulfate salt.
26. The kit of claim 25, wherein the ionic detergent is lithium lauryl sulfate or sodium dodecyl sulfate.
- 30 27. The kit of any one of claims 24 to 26, wherein the ionic detergent is at a final concentration of between about 0.1-0.7% (w/v).
28. The kit of any one of claims 24 to 27, wherein the second reagent or the amplification reagent further comprises metal ions necessary for an enzymatic reaction.
- 35 29. The kit of any one of claims 24 to 28, wherein the non-ionic detergent is a polyoxyethylene sorbitan monoalkylate derivative or a polyoxyethylene p-t-octyl phenol derivative.
- 40 30. The kit of claim 30, wherein the non-ionic detergent is polyoxyethylene (20) sorbitan monolaurate, polyoxyethylene (20) sorbitan monooleate, polyoxyethylene (20) sorbitan monopalmitate, polyoxyethylene (20) sorbitan monostearate or a polyoxyethylene (9) p-t-octyl phenol derivative.
31. The kit of any one of claims 24 to 30, further comprising an RNase-inhibiting agent.
- 45 32. The kit of claims 31, wherein the RNase-inhibiting agent is a protease.
33. The kit of claim 32, wherein the protease is proteinase K.
34. The kit of any one of claims 24 to 33, wherein the amplification reagent contains at least one primer capable of hybridizing to a sequence specific to a ribosomal RNA.
- 50 35. The kit of claim 34, wherein the primer is capable of hybridizing to a sequence specific to a ribosomal RNA derived from *Chlamydia trachomatis*, *Ureaplasma urealyticum* or human cells infected with Human Papilloma Virus.

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#### Patentansprüche

1. Ein Verfahren zur Erhöhung enzymatischer Aktivität in einer Flüssigkeit, die ein ionisches Detergens und minde-

stens ein Enzym-Substrat enthält, aufweisend folgende Schritte :

- a. Vorsehen einer Flüssigkeit, die mindestens ein ionisches Detergens enthält, das in einer Menge vorhanden ist, die ausreichend ist, um eine enzymatische Aktivität zu hemmen, und mindestens ein Enzym-Substrat;
  - 5 b. Mischen eines nicht-ionischen Detergenses in die Flüssigkeit zu einer Endkonzentration zwischen etwa 6-20% (v/v) des nicht-ionischen Detergenses, wobei eine Mischung hergestellt wird, und wobei ein hemmender Effekt des ionischen Detergenses auf die enzymatische Aktivität unterdrückt wird;
  - c. Zugabe zur Mischung mindestens eines Enzyms, das die enzymatische Aktivität besitzt; und
  - 10 d. Inkubieren der Mischung unter Reaktionsbedingungen, die für die enzymatische Aktivität günstig sind.
2. Das Verfahren gemäß Anspruch 1, wobei die Flüssigkeit eine biologische Probe aufweist.
  3. Das Verfahren gemäß Anspruch 1 oder 2, wobei das Enzym-Substrat eine Nukleinsäure aufweist.
  - 15 4. Das Verfahren nach einem der Ansprüche 1 bis 3, wobei das Enzym aus der Gruppe ausgewählt ist, die aus einer DNA-gesteuerten DNA-Polymerase, einer RNA-gesteuerten DNA-Polymerase, einer DNA-gesteuerten RNA-Polymerase, einem RNA-hydrolysierenden Enzym, einer Restriktions-Endonuklease und einer Protease besteht.
  5. Das Verfahren gemäß Anspruch 4, wobei das Enzym aus der Gruppe ausgewählt ist, das einer Reverse-Transkriptase, die von einem Retrovirus abgeleitet ist, einer DNA-Polymerase, die von *Thermus aquaticus* oder von *Bacillus stearothermophilus* abgeleitet ist, einer Restriktions-Endonuklease und einer T7 RNA-Polymerase besteht.
  - 20 6. Das Verfahren nach einem der Ansprüche 1 bis 5, wobei das ionische Detergens eine Zusammensetzung aufweist, die aus der Gruppe ausgewählt ist, die aus wasserlöslichen Laurylsulfatsalzen besteht.
  7. Das Verfahren nach Anspruch 6, wobei das ionische Detergens Lithiumlaurylsulfat oder Natriumdodecylsulfat ist.
  8. Das Verfahren nach einem der Ansprüche 1 bis 7, wobei die Endkonzentration des ionischen Detergenses zwischen 0,1% und 0,7% (w/v) beträgt.
  - 30 9. Das Verfahren nach einem der Ansprüche 1 bis 8, wobei das nicht-ionische Detergens ein Derivat von Polyoxyethylen (20) Sorbitanmonoalkylat oder ein Derivat von Polyoxyethylen p-t-Octylphenol ist.
  10. Das Verfahren gemäß Anspruch 9, wobei das nicht-ionische Detergens ein Derivat von Polyoxyethylen (20) Sorbitanmonolaurat, Polyoxyethylen (20) Sorbitanmonopalmitat, Polyoxyethylen (20) Sorbitanmonostearat, Polyoxyethylen (20) Sorbitanmonooleat oder Polyoxyethylen (9) p-t-Octylphenol ist.
  - 35 11. Ein Verfahren zum Detektieren einer Nukleinsäure in einer biologischen Probe, das folgende Schritte aufweist:
  - 40 a) Kontaktieren einer biologischen Probe, die eine Zielnukleinsäuresequenz enthält, mit einem ionischen Detergens;
  - b) dann Mischen eines nicht-ionischen Detergenses bei einer Endkonzentration von zwischen 6-20% (v/v) mit der biologischen Probe, wodurch ein hemmender Effekt des ionischen Detergenses auf enzymatische Aktivität
  - 45 unterdrückt wird;
  - c) Zugabe mindestens eines Nukleinsäure-Primers, der eine Nukleinsäuresequenz, die imstande ist, zu der Zielnukleinsäuresequenz zu hybridisieren, Nukleotidtriphosphate, erforderliche Cofaktoren oder Salze und mindestens ein Enzym hat, das eine Nukleinsäure-Polymerase Aktivität hat, wodurch eine Mischung hergestellt wird ;
  - 50 d) Inkubieren der Mischung unter Bedingungen, die ausreichend sind, um die Amplifizierung der Zielnukleinsäuresequenz zu bewirken, wodurch eine amplifizierte Zielnukleinsäuresequenz hergestellt wird; und
  - e) Detektieren der amplifizierten Zielnukleinsäuresequenz.
  12. Das Verfahren gemäß Anspruch 11, wobei das ionische Detergens eine Zusammensetzung aufweist, die aus der Gruppe ausgewählt ist, die aus den wasserlöslichen Laurylsulfatsalzen besteht.
  - 55 13. Das Verfahren gemäß Anspruch 12, wobei das ionische Detergens Lithiumlaurylsulfat oder Natriumdodecylsulfat ist.



14. Das Verfahren gemäß einem der Ansprüche 11 bis 13, wobei die Endkonzentration des ionischen Detergenses zwischen etwa 0,1-0,7% (w/v) beträgt.
- 5 15. Das Verfahren gemäß einem der Ansprüche 11 bis 14, wobei das nicht-ionische Detergens eine Zusammensetzung aufweist, die aus der Gruppe ausgewählt ist, die aus Polyoxyethylen (20) Sorbitanmonoalkylat-Derivaten und Polyoxyethylen p-t-Octylphenol-Derivaten besteht.
- 10 16. Das Verfahren gemäß Anspruch 15, wobei das nicht-ionische Detergens Polyoxyethylen (20) Sorbitanmonolaurat, Polyoxyethylen (20) Sorbitanmonooleat, Polyoxyethylen (20) Sorbitanmonopalmitat, Polyoxyethylen (20) Sorbitanmonostearat oder ein Derivat von Polyoxyethylen (9) p-t-Octylphenol ist.
17. Das Verfahren gemäß einem der Ansprüche 11 bis 16, wobei die Zielnukleinsäuresequenz mindestens eine Sequenz aufweist, die für ribosomale RNA oder ribosomale DNA spezifisch ist.
- 15 18. Das Verfahren gemäß Anspruch 17, wobei die ribosomale RNA-Sequenz für *Chlamydia trachomatis*, *Ureaplasma urealyticum* oder menschliche Zellen, die mit dem menschlichen Papillomvirus infiziert worden sind, spezifisch ist.
19. Das Verfahren gemäß Anspruch 11, wobei der Schritt des Mischens die Zugabe des nicht-ionischen Detergenses simultan mit oder vor der Zugabe von Metallionen, die für die enzymatische Reaktion erforderlich sind, vorsieht.
- 20 20. Das Verfahren gemäß einem der Ansprüche 11 bis 19, wobei der Schritt des Zugebens ferner die Zugabe eines die RNase inhibierenden Agens und, gegebenenfalls, das Inaktivieren des die RNase hemmenden Agenses vor der Zugabe mindestens eines Enzyms, das Nukleinsäure-Polymerase-Aktivität besitzt, aufweist.
- 25 21. Das Verfahren gemäß Anspruch 20, wobei das die RNase hemmende Agens eine Protease ist.
22. Das Verfahren gemäß Anspruch 21, wobei die Protease Protinase K ist.
23. Das Verfahren gemäß einem der Ansprüche 20 bis 22, wobei der Schritt zur Inaktivierung des die RNase inhibierenden Agenses das Erwärmen der Mischung bis auf zwischen etwa 90°C bis 100°C für zwischen etwa 1 bis 5 Minuten aufweist.
- 30 24. Ein Kit zur Amplifizierung einer Zielnukleinsäure in einer biologischen Probe, wobei der Kit aufweist:
  - 35 a) ein erstes Reagens zum Sammeln und zum Überführen einer biologischen Probe, die eine Zielnukleinsäuresequenz enthält, wobei das Reagens ein ionisches Detergens bei einer Endkonzentration von zwischen etwa 0,1%-1,5% (w/v) aufweist;
  - b) ein zweites Reagens, das ein nicht-ionisches Detergens zum Mischen mit dem ersten Reagens enthält, so daß die Endkonzentration des nicht-ionischen Detergenses zwischen etwa 6-20% (v/v) beträgt;
  - 40 c) ein Amplifizierungsreagens, das mindestens einen Primer aufweist, der imstande ist, zu der Zielnukleinsäuresequenz zu hybridisieren, Nukleotid-Triphosphate und Cofaktoren oder Salze, die für die Nukleinsäure-Amplifizierung erforderlich sind; und
  - d) mindestens ein Enzym, das Nukleinsäure-Polymerase-Aktivität besitzt, zum Initiieren der Nukleinsäure-Amplifizierung der Zielnukleinsäuresequenz.
- 45 25. Das Kit gemäß Anspruch 24, wobei das ionische Detergens ein Laurylsulfatsalz ist.
26. Das Kit gemäß Anspruch 25, wobei das ionische Detergens Lithiumlaurylsulfat oder Natriumdodecylsulfat ist.
- 50 27. Das Kit gemäß einem der Ansprüche 24 bis 26, wobei die Endkonzentration des ionischen Detergenses zwischen etwa 0,1-0,7% (w/v) beträgt.
28. Das Kit gemäß einem der Ansprüche 24 bis 27, wobei das zweite Reagens oder das Amplifikations-Reagens ferner Metallionen, die für eine enzymatische Reaktion erforderlich sind, aufweist.
- 55 29. Das Kit gemäß einem der Ansprüche 24 bis 28, wobei das Kit das nicht-ionische Detergens ein Derivat von Polyoxyethylen-Sorbitanmonoalkylat oder ein Derivat von Polyoxyethylen p-t-Octylphenol ist.

30. Das Kit gemäß Anspruch 30, wobei das nicht-ionische Detergens Polyoxyethylen (20) Sorbitanmonolaurat, Polyoxyethylen (20) Sorbitanmonooleat, Polyoxyethylen (20) Sorbitanmonopalmitat, Polyoxyethylen (20) Sorbitanmonostearat oder ein Derivat von Polyoxyethylen (9) p-t-Octylphenol ist.

31. Das Kit gemäß einem der Ansprüche 24 bis 30, weiter aufweisend ein die RNase inhibierendes Agens.

32. Das Kit gemäß Anspruch 31, wobei das die RNase inhibierende Agens eine Protease ist.

33. Das Kit gemäß Anspruch 32, wobei die Protease Proteinase K ist.

34. Das Kit gemäß einem der Ansprüche 24 bis 33, wobei das Amplifizierungs-Reagens mindestens einen Primer enthält, der imstande ist, zu einer Sequenz zu hybridisieren, die spezifisch für eine ribosomale RNA ist.

35. Das Kit gemäß Anspruch 34, wobei der Primer imstande ist, zu einer Sequenz zu hybridisieren, die spezifisch für eine ribosomale RNA ist, die von *Chlamydia trachomatis*, *Ureaplasma urealyticum* oder menschlichen Zellen, die mit dem menschlichen Papillomvirus infiziert sind, abgeleitet ist.

## Revendications

1. Une méthode pour augmenter l'activité enzymatique dans un liquide contenant un détergent ionique et au moins un substrat d'enzyme, comprenant les étapes de :

(a) fourniture d'un liquide contenant au moins un détergent présent dans une quantité suffisante pour inhiber une activité enzymatique et au moins un substrat d'enzyme ;

(b) mélange d'un détergent non-ionique dans le liquide à une concentration finale entre environ 6-20 % (en volume/volume) dudit détergent non-ionique, de façon à produire un mélange et supprimer un effet inhibiteur du détergent ionique sur l'activité enzymatique ;

(c) addition au mélange d'au moins une enzyme ayant l'activité enzymatique ; et

(d) incubation du mélange selon des conditions de réaction favorables à l'activité enzymatique.

2. La méthode selon la revendication 1, dans laquelle le liquide comprend un échantillon biologique.

3. La méthode selon la revendication 1 ou 2, dans laquelle le substrat d'enzyme comprend un acide nucléique.

4. La méthode selon l'une quelconque des revendications 1 à 3, dans laquelle est choisie dans le groupe constitué par une polymérase d'ADN dirigée vers l'ADN, une polymérase d'ADN dirigée vers l'ARN, une polymérase d'ARN dirigée vers l'ADN, une enzyme hydrolysant l'ARN, une endonucléase de restriction et une protéase.

5. La méthode selon la revendication 4, dans laquelle l'enzyme est choisie parmi le groupe constitué par une transcriptase inverse provenant de rétrovirus, une polymérase d'ADN provenant de *Thermus aquaticus* ou de *Bacillus stearothermophilus*, une endonucléase de restriction et une polymérase d'ARN T7.

6. La méthode selon l'une quelconque des revendications 1 à 5, dans laquelle le détergent ionique comprend une composition choisie dans le groupe constitué par les sels de laurylsulfate solubles dans l'eau.

7. La méthode selon la revendication 6, dans laquelle le détergent ionique est le laurylsulfate de lithium ou le sulfate de dodécylsodium.

8. La méthode selon l'une quelconque des revendications 1 à 7, dans laquelle le détergent ionique est à une concentration finale d'environ 0,1 % à 0,7 % (en poids/volume).

9. La méthode selon l'une quelconque des revendications 1 à 8, dans laquelle le détergent non ionique est un dérivé de polyoxyéthylène (20) sorbitan monoalkylé ou un dérivé de polyoxyéthylène p-t-octylphénol.

10. La méthode selon la revendication 9, dans laquelle le détergent non-ionique est un dérivé polyoxyéthylène (20) sorbitan monolaurate, polyoxyéthylène (20) sorbitan monopalmitate, polyoxyéthylène (20) sorbitan monostéarate, polyoxyéthylène (20) sorbitan mono-oléate ou polyoxyéthylène (9) p-t-octylphénol.

11. Une méthode pour détecter un acide nucléique dans un échantillon biologique, comprenant les étapes de :

- a) mise en contact d'un échantillon biologique contenant une séquence d'acide nucléique cible avec un détergent ionique ; puis
- 5 b) mélange d'un détergent non-ionique à une concentration finale d'environ 6 à 20 % (en volume/volume) avec l'échantillon biologique, supprimant de ce fait un effet inhibiteur du détergent ionique sur l'activité enzymatique ;
- c) addition d'au moins une amorce d'acide nucléique ayant une séquence d'acide nucléique capable de s'hybrider à la séquence d'acide nucléique cible, nucléotide triphosphates, cofacteurs ou sels nécessaires, et d'au moins une enzyme ayant l'activité de polymérase d'acide nucléique, de manière à produire un mélange ;
- 10 d) incubation du mélange selon des conditions suffisantes pour provoquer l'amplification de la séquence d'acide nucléique cible, de manière à produire une séquence d'acide nucléique cible amplifiée ; et
- e) détection de la séquence d'acide nucléique cible amplifiée.

12. La méthode selon la revendication 11, dans laquelle le détergent ionique comprend une composition choisie dans le groupe constitué par les sels de laurylsulfate solubles dans l'eau.

13. La méthode selon la revendication 12, dans laquelle le détergent ionique est le lithium laurylsulfate ou le sodium dodécylsulfate.

14. La méthode selon l'une quelconque des revendications 11 à 13, dans laquelle le détergent non ionique est à une concentration finale d'environ 0,1 à 0,7 % (en poids/volume).

15. La méthode selon l'une quelconque des revendications 11 à 14, dans laquelle le détergent non-ionique comprend une composition choisie dans le groupe constitué par les dérivés de polyoxyéthylène (20) sorbitan monoalkylé et les dérivés de polyoxyéthylène p-t-octylphénol.

16. La méthode selon la revendication 15, dans laquelle le détergent non-ionique est un dérivé de polyoxyéthylène (20) sorbitan monolaurate, polyoxyéthylène (20) sorbitan monooléate, polyoxyéthylène (20) sorbitan monopalmitate, polyoxyéthylène (20) sorbitan monostéarate ou polyoxyéthylène (9) p-t-octylphénol.

17. La méthode selon l'une quelconque des revendications 11 à 16, dans laquelle la séquence d'acide nucléique cible comprend au moins une séquence spécifique à l'ARN ribosomique ou l'ADN ribosomique.

18. La méthode selon la revendication 17, dans laquelle la séquence d'ARN ribosomique est spécifique des cellules de *Chlamydia trachomatis*, *Urea-plasma urealyticum* ou cellules humaines infectées par le virus de Papillome humain.

19. La méthode selon la revendication 11, dans laquelle l'étape de mélange comprend l'addition du détergent non-ionique de façon simultanée ou précède l'addition d'ions métalliques nécessaires à la réaction enzymatique.

20. La méthode selon l'une quelconque des revendications 11 à 19, dans laquelle l'étape d'addition comprend de plus l'addition d'un agent inhibiteur d'ARNase, et, de façon optionnelle, l'inactivation de l'agent inhibiteur d'ARNase avant l'addition d'au moins une enzyme ayant une activité de polymérase d'acide nucléique.

21. La méthode selon la revendication 20, dans laquelle l'agent inhibiteur d'ARNase est une protéase.

22. La méthode selon la revendication 21, dans laquelle la protéase est la protéase K.

23. La méthode selon l'une quelconque des revendications 20 à 22, dans laquelle l'étape d'inactivation de l'agent inhibiteur d'ARNase comprend le chauffage du mélange à environ 90°C à 100°C pendant environ 1 à 5 minutes.

24. Un kit pour amplifier un acide nucléique cible dans un échantillon biologique, ledit kit comprenant :

- a) un premier réactif pour collecter et transporter un échantillon biologique contenant une séquence d'acide nucléique cible, comprenant un détergent ionique à une concentration finale d'environ 0,1 % à 1,5 % (en poids/volume) ;
- 55 b) un second réactif contenant un détergent non-ionique pour être mélangé avec le premier réactif de telle sorte que le détergent non-ionique soit à une concentration finale d'environ 6 à 20 % (en volume/volume) ;

c) un réactif d'amplification comprenant au moins une amorce capable de s'hybrider à la séquence d'acide nucléique cible, aux nucléotide triphosphates et cofacteurs ou sels nécessaires pour l'amplification d'acide nucléique ; et

d) au moins une enzyme ayant l'activité de polymérase d'acide nucléique pour amorcer l'amplification d'acide nucléique d'une séquence d'acide nucléique cible.

25. Le kit selon la revendication 24, dans lequel le détergent ionique est un sel de laurylsulfate.

26. Le kit selon la revendication 25, dans lequel le détergent ionique est le sulfate de lithium lauryle ou le sulfate de sodium dodécyle.

27. Le kit selon l'une quelconque des revendications 24 à 26, dans lequel le détergent ionique est à une concentration finale d'environ 0,1 à 0,7 % (en poids/volume).

28. Le kit selon l'une quelconque des revendications 24 à 27, dans lequel le second réactif ou le réactif d'amplification comprend de plus des ions métalliques nécessaires à la réaction enzymatique.

29. Le kit selon l'une quelconque des revendications 24 à 28, dans lequel le détergent non-ionique est un dérivé de polyoxyéthylène sorbitan monoalkylé ou un dérivé polyoxyéthylène p-t-octylphénol.

30. Le kit selon la revendications 29, dans lequel le détergent non-ionique est un dérivé de polyoxyéthylène (20) sorbitan monolaurate, polyoxyéthylène (20) sorbitan mono-oléate, polyoxyéthylène (20) sorbitan monopalmitate, polyoxyéthylène (20) sorbitan monostéarate ou polyoxyéthylène (9) p-t-octylphénol.

31. Le kit selon l'une quelconque des revendications 24 à 30, comprenant de plus un agent inhibiteur d'ARNase.

32. Le kit selon la revendication 31, dans lequel l'agent inhibiteur d'ARNase est une protéase.

33. Le kit selon la revendication 32, dans lequel la protéase est la protéinase K.

34. Le kit selon l'une quelconque des revendications 24 à 33, dans lequel le réactif d'amplification contient au moins une amorce capable de s'hybrider à la séquence spécifique de l'ARN ribosomique.

35. Le kit selon la revendication 34, dans lequel l'amorce est capable de s'hybrider à une séquence spécifique de l'ARN ribosomique provenant de *Chlamydia trachomatis*, *Ureaplasma urealyticum* ou cellules humaines infectées par le virus de Papillome humain.

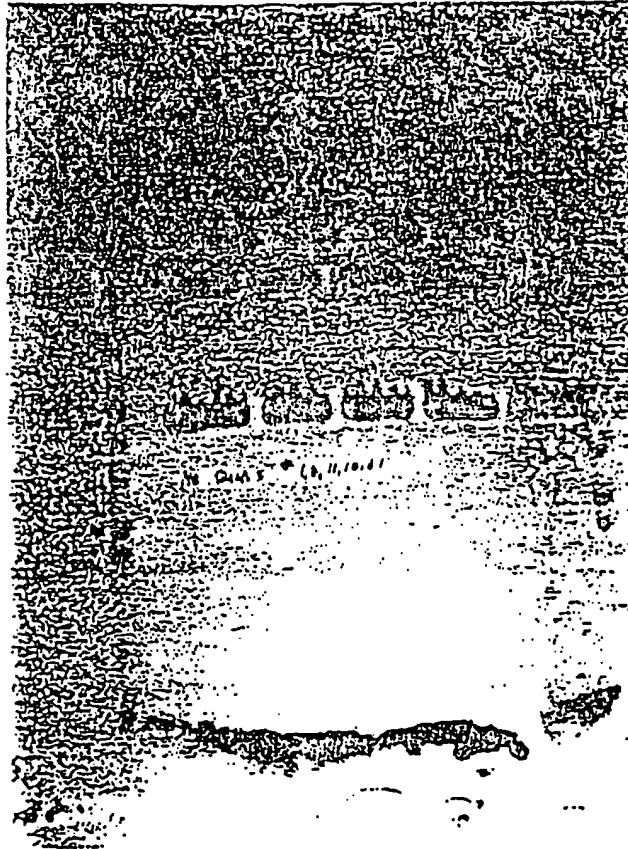


FIGURE 1